Tail and fin rot bacteria in Icelandic aquaculture

Diversity and genetic analysis

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Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

Sporð- og uggarots bakteríur í íslensku fiskeldi

Fjölbreytileiki og erfðatæknileg greining

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Ágrip

Sporð- og uggarot hefur greinst í auknum mæli í fiskeldisstöðvum á Íslandi. Einkenni sjúkdómsins eru einna helst opin sár, vefjaskemmdir og rot á sporði og uggum en einkennin geta verið mis alvarleg allt frá litabreytingum á roði yfir í kerfisbundnar sýkingar. Orsök sjúkdómsins eru sýkingar af völdum *Flavobacterium* og *Tenacibaculum* bakteríutegunda og í ritgerðinni eru fimm slíkar tegundir teknar til skoðunar; *Flavobacterium psychrophilum, Flavobacterium columnare, Flavobacterium branchiophilum, Tenacibaculum maritimum* og *Tenacibaculum soleae*.

Bakteríurnar flokkast sem Gram neikvæðir stafir og mynda föl-gular og gular þyrpingar á *Flexibacter maritimus* medium (FMM) agarskálum. Erfiðlega hefur reynst að staðfesta sýkingar með ræktun vegna þess að bakteríurnar eru hægvaxta, og því geta hraðvaxta umhverfisbakteríur vaxið yfir hina eiginlegu sjúkdómsvalda. Til þess að bæta greiningu á þessum bakteríum voru tegundasértækir polymerase chain reaction (PCR) vísar og DNA raðgreining notuð.

Markmið rannsóknarinnar var að einangra sjúkdómsvaldandi sporð- og uggarots bakteríur úr eldisfiski og viltum fiski úr sjó og tegundagreina bakteríurnar með sameindalíffræðilegum aðferðum. Annað markmið var að kanna stofnabreytileika íslenskra *Flavobacterium* stofna og útbúa skyldleikatré með samanburði við viðmiðunarstofna. Að lokum voru vaxtarskilyrði viðmiðunarstofna sporð- og uggarotsbaktería athuguð, auk bakteríustofna sem einangraðir voru úr sjúkum fiskum hér á landi. Gul-leitar bakteríuþyrpingar sem líktust sporð- og uggarots bakteríum agarskálum voru greindar í ljóssmásjá og Gram litaðar. Þar á eftir voru bakteríur greindar með völdum tegundasértækum PCR vísum sem bindast á svæði 16S rRNA gens bakteríanna sem síðar var staðfest með raðgreiningu 16S rRNA gensins. Raðir 16S rRNA gena úr íslenskum *Flavobacterium* stofnum voru bornar saman við viðmiðunarstofna *F. psychrophilum, T. maritimum* og *T. soleae* og útfrá því útbúið “Neighbor-Joining” skyldleikatré. Út frá skyldleikatrénu má sjá að innan íslensku *Flavobacterium* stofnanna eru hópar af skyldum stofnum, en þegar á heildina er litið þá eru íslensku stofnarnir fjölbreyttir að arfgerð og margir hverjir ólíkir *F. psychrophilum v*iðmiðunarstofninum. Þó má greina nokkra stóra hópa baktería sem hópast með viðmiðunarstofninum.

Fjölda tegundasértækra vísa hefur verið lýst fyrir sporð- og uggarots bakteríur. Þegar tegundasértækir vísar, sem birtir höfðu verið í nokkrum vísindagreinum, voru prófaðir kom fljótlega í ljós að sértækni greiningarvísanna var ófullnægjandi. Niðurstöður raðgreininganna bentu til að mismunandi tegundir sporð- og uggarots baktería sýndu nægilegan breytileika innan 16S rRNA gensins og 16S – 23S milligena svæðisins til þess að hægt væri að hanna sértækari greiningarvísa fyrir sporð- og uggarots bakteríutegundir.

Að lokum var vöxtur viðmiðunarstofna auk nokkurra sérílslenskra sporð- og uggarots stofna kannaður með tilliti til mismunandi seltu- og hitastigs. Eins og við mátti búast óx *F. psychrophilum* best við ferskvatnsaðstæður (0 ppm), en vöxtur greindinst einnig við önnur seltu seltustig. *T. maritimum, T. soleae* og bakteríustofnar einangraðir úr fiski úr sjó eða ræktaðir við seltu uxu best í æti með fullri seltu (32 ppm) en uxu þó einnig í æti með hálfri seltu (16 ppm). Viðmiðunarstofnar og allir íslensku stofnarnir uxu best við 15°C, en það var hæsta hitastig sem tilraunin bauð uppá.

Abstract

Tail and fin rot, caused by *Flavobacterium* and *Tenacibaculum* bacteria, is an emerging disease worldwide that has recently been detected in Icelandic aquaculture. Infected fish can display open lesions, tissue damage and tail and fin rot. The symptoms can vary in severity from metachromatic skin to systemic infection. In this study, pathogenic bacteria of fish in the genera *Flavobacterium* and *Tenacibaculum* were studied; *Flavobacterium psychrophilum, Flavobacterium columnare, Flavobacterium branchiophilum, Tenacibaculum maritimum* and *Tenacibaculum soleae*.

The cells are Gram-negative, slender rods that appear pale yellow or yellow on agar plates. Identification of the etiological agent can be difficult when based solely on bacterial culture, as the *Flavobacterium* and *Tenacibaculum* species are slow growing bacteria that are easily overgrown by contaminating bacteria. To improve the power of identification, polymerase chain reaction (PCR) and DNA sequencing were applied.

The objective of this study was to isolate the etiological agents of tail and fin rot from diseased Icelandic fish, both farmed and wild caught, and analyze them using molecular analysis. Yellow pigmented colonies that resembled tail and fin rot bacteria were examined under a light microscope and Gram-stained, after which the 16S rRNA gene was amplified and sequenced to confirm the bacterial identity. The 16S rRNA gene sequences of the Icelandic isolates were furthercompared to those of published isolates by phylogenetic analysis. The Neighbor-Joining phylogenetic tree displayed great variance within many of the Flavobacterium isolates in Iceland and many isolates prove to be unrelated to the *F. psychrophilum* reference strain. Some isolates, however, group together with the reference strain.

Numerous primers have been published that are supposedly species-specific. However, upon further examination, species-non-specific amplification appears to be common. Although the *Flavobacterium* and *Tenacibaculum* species are related, comparison of the 16S rRNA gene and 16S – 23S IGS region of numerous isolates revealed unique regions, which led us to believe that more species-specific primers could be developed.

Finally, bacterial growth of reference strains and isolates collected from diseased fish in Iceland was examined to test the temperature and salinity tolerance.

As expected, *Flexibacter maritimus* medium (FMM) medium containing freshwater (0 ppm) was best suited for *F. psychrophilum* growth, although growth at other salinity levels was also noted. FMM containing 32 ppm (seawater) was most effective for *T. maritimum* and *T. soleae* and isolates from fish caught at sea and fish grown in saline water, although they were also able to grow in brackish water.. The optimal temperature for all bacterial species was 15°C, which was the highest temperature used in this study.

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AOA	Anacker and Ordal agar
BCWD	Bacterial cold water disease
CFB	Cytophaga-Flavobacterium-Bacteroides
ECP	Extracellular products
FMM	Flexibacter maritimus medium
GyrB	Gyrase B subunit
IFAT	Indirect fluorescent antibody technique
MA	Marine agar
MSS	Minimal salt solution
MLST	Multilocus sequence typing
OD	Optical density
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
RTFS	Rainbow trout fry syndrome
RBL	Rhamnose-binding lectin
IGS	Intergenic spacer

1 Introduction

1.1 Flavobacterial diseases

Flavobacterial diseases, caused by members of the *Flavobacterium* and *Tenacibaculum* genera, are considered to be a big threat to both wild and farmed fish (1) both economically and ecologically. For nearly 100 years, scientific research has gone on for prevention and control of flavobacterial diseases. This has been complicated by difficulty isolating and culturing the etiological agents, as well as reproducing disease in experimental challenge models to study pathogenicity (2).

Three species in the Flavobacterium genus; *Flavobacterium psychrophilum*, *Flavobacterium columnare* and *Flavobacterium branchiophilum*, and two species in the *Tenacibaculum* genus, *Tenacibaculum soleae* and *Tenacibaculum maritimum*, are considered to be important pathogens of fish (2-4). In addition, *Tenacibaculum finnmarkense* is a newly discovered pathogen in Norwegian sea-reared Atlantic salmon (5), which underscores the importance of screening for new, pathogenic species.

Disease outbreaks linked to tail and fin rot bacteria have increased in Iceland since 2012 (6). As infections caused by tail and fin rot bacteria in Iceland have only been confirmed using morphological characteristics, this study focuses on using molecular biology for identification and classification of flavobacterial species.

1.2 Flavobacteriaceae

The family Flavobacteriaceae consists of a diverse group of bacteria that belong to the phylum Cytophaga-Flavobacterium-Bacteroides (CFB). The phylogenetic relationship of species within Flavobacteriaceae has been described using 16S rRNA gene sequencing and DNA-rRNA hybridization, as well as gyrase B subunit (*gyrB)* sequencing. Figure 1 shows the relationship within the family of Flavobacteriaceae (7).

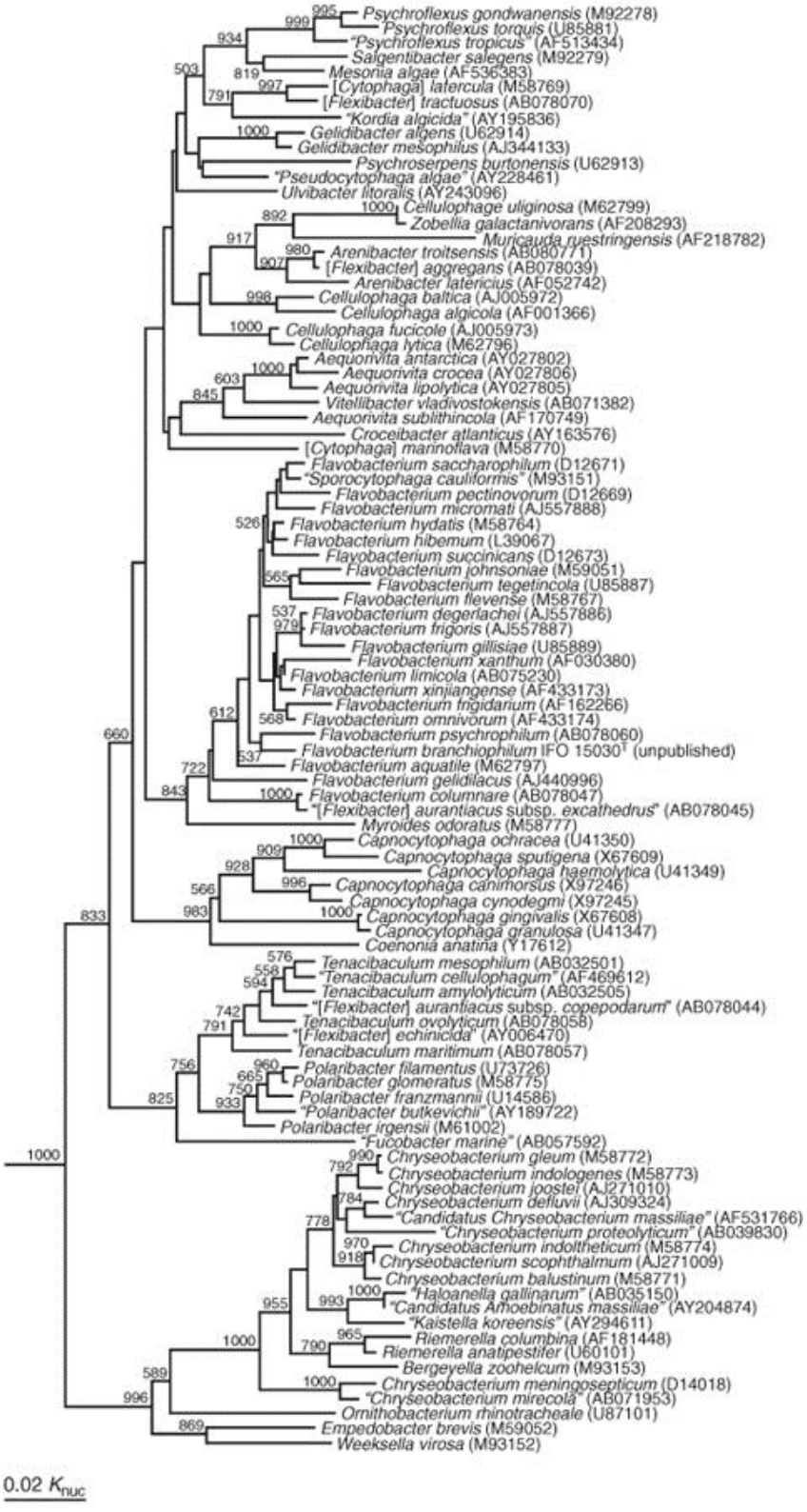


Figure 1. Relationship between species within the Flavobacteriaceae family.
Relationship of species within the family Flavobacteriaceae, based on 16S rRNA gene sequence analysis using neighbor-joining method with 1,000 bootstrap replications. Each genus is represented by a type strain, with the accession number in brackets. The scale bar is 0.02 K_{nuc}. The numbers by the branches show the confidence limits estimated by bootstrap analysis. The tree includes all species of Flavobacteriaceae except *Myroides odoratimimus* and *Tenacibaculum soleae,* for which no data was available (7).

1.3 Flavobacterium psychrophilum

F. psychrophilum was initially described as Cytophaga psychrophila based on biochemical characteristics in 1960. Later it was reclassified as *Flexibacter psychrophilus* based on DNA homology but finally it gained its current classification as *F. psychrophilum* based on DNA-rRNA hybridization data in 1996 (8-11).

1.3.1 Morphology and physiological characteristics

The Gram-negative bacterium *F. psychrophilum* is strictly aerobic yellow-pigmented, flexible and rod-shaped with rounded ends (8, 12). The cells are approximately 1 to 12 µm long and 0.3 to 0.5 µm wide, although shorter and wider rods have been detected (13, 14). Some *F. psychrophilum* strains have gliding motility while other strains lack this trait (8).

F. psychrophilum produces catalase and oxidase but does not generate H_2S, indole, lysine or ornithine decarboxylase. Gelatin, casein, and tributyrin are degraded but nitrates are not reduced. The amino acid tyrosine is degraded by some isolates but some chemicals are not, e.g. chitin, aesculin, starch and xanthine. The G+C ratio of the DNA ranges from 32.5 to 34.0 mol% (14, 15).

1.3.2 Bacterial culture and isolation

The detection and isolation of *F. psychrophilum* is normally based on culture on solid media. It is a slow growing freshwater bacterium that thrives at relatively cold temperatures; growing at temperatures between 4°C and 23°C, with optimal growth between 15°C and 20°C (11, 13). Moreover, the salinity tolerance of *F. psychrophilum* growth is limited to 0.5% to 2.0% NaCl (13, 14). Isolation of *F. psychrophilum* is difficult, as it is easily overgrown by faster growing environmental bacteria (16).

Cepeda *et al.* compared different media used for isolation and characterization of *F. psychrophilum* and found that the most successful medium was a modified version of Anacker and Ordal agar (AOA) (17) which was modified with tryptone, salts and glucose (18). Although a modified version of the minimal medium AOA is considered more successful for the growth of *F. psychrophilum*, AOA medium and other minimal media, such as FMM that is without glucose and mainly contains peptone, have proven to be productive in culturing the bacteria (19).

1.3.3 Virulence factors

Madetoja *et al.* isolated *F. psychrophilum* from rainbow trout broodfish lesions, but were also able to isolate the bacterium from the intestines of apparently healthy fish and from tank water, which the fish were kept in. It is possible that the bacterial strains in the intestines and water were of low virulence or that the bacterial density was too low to cause disease. However Madetoja *et al*. concluded that subclinically infected rainbow trout broodfish might be a source of infection (20).

F. psychrophilum can enter hosts through injured skin. Miwa and Nakayasu demonstrated this in a challenge study where ayu fish were infected with *F. psychrophilum* using intramuscular injection. At the injection site, damaged skin developed both ulcers and hemorrhagic lesion (21).

F. psychrophilum encodes 13 allegedly secreted proteases that are believed to be involved in the destruction of host tissue (15). Fpp1 and Fpp2 are thermo-labile cold-adapted metalloproteases that

lose up to 50% of their activity at 18°C. However, calcium increases their thermostability at high temperatures (22, 23). Both Fpp1 and Fpp2 show maximal activity at pH 6.5, but Fpp2 has a broader range of pH activity than Fpp1 (22, 23). The Fpp1 and Fpp2 ability to degrade all protein elements of connective tissue suggests a role in the invasion of tissue during the process of infection. The expression of Fpp1 is dependent on calcium concentration, which underscores the importance of calcium within the host during invasion of *F. psychrophilum.* Incubation at 5°C to 10°C resulted in an increase in Fpp1 levels, which correlated with the development of bacterial cold water disease (BCWD) (22, 23).

Other metalloproteases have been identified using whole genome sequencing of *F. psychrophilum* as well as collagenase-encoding genes (15). Ostland *et al*. suggested that extracellular collagenases play an important role in the virulence of *F. psychrophilum*, while Duchaud *et al*. showed that the collagenase-encoding genes in *F. psychrophilum* are disrupted by an insertion sequence. The role of collagenases in *F. psychrophilum* virulence remains to be fully explored (15, 24).

1.3.4 Bacterial coldwater disease (BCWD)

F. psychrophilum is known to cause Bacterial cold water disease (BCWD), also known as rainbow trout fry syndrome (RTFS) (8, 25) and is a significant threat to salmonid farming around the world (26). It is likely that *F. psychrophilum* can cause disease in all salmonid fish species. The bacterium has also been isolated from non-salmonid fish, such as the Japanese eel (*Anguilla japonica),* European eel (*Anguilla anguilla*), tench (*Tinca tinca*), pale chub (*Zaco platypus*), perch (*Parca fluviatilis*), roach (*Rutilis rutilis*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*) and ayu (*Plecoglossus altivelis*). It has a wide distribution and has been found in North America, Japan, Korea, Australia, Chile and various European countries, including France, Germany, Denmark, United Kingdom, Spain, Finland, Italy, Belgium, and Iceland (unpublished results) (8, 25).

1.3.5 Clinical signs

Salmonid fry infected with *F. psychrophilum* can display erosion of the peduncle area and the spinal cord that can lead to tail loss. Fry that have overcome infection may grow up to have abnormal swimming behavior and spinal malformation. Diseased yearlings can develop lesions around the peduncle area and anterior to the dorsal fin. Some show lesions on the lower jaw and close to the anal area. Lesions may also be involved in subsequent systemic infection. Less common signs are local anemia, cephalic osteochondritis, exophthalmia, hemorrhaging of the gills, necrotic myositis and necrotic scleritis (8, 12).

1.4 Flavobacterium columnare

The column-like structure of *F. columnare* was the reason for its first name, *Bacillus columnaris,* and the name of the disease: columnaris disease. Later *F. columnare* was named *Chondrococcus columnaris*, *Cytophaga columnaris* and *Flexibacter columnaris* but finally its current name *Flavobacterium columnare* is based on DNA-rRNA hybridization data and protein and fatty acid profiles (11, 27).

1.4.1 Morphology and physiological characteristics

This Gram-negative bacterium was first described by Davis in 1922 (1). Since then, this species has undergone taxonomic changes but is presently referred to as *Flavobacterium columnare* (28). *F. columnare* has been described as a rod shaped, Gram-negative cell with parallel or irregular sides. The cells are generally 2 to 5 µm long and 0.3 to 0.5 µm wide and can glide with gradual motion without flagella (28, 29).

F. columnare is able to degrade gelatin and casein without acid production. It can hydrolyze various polysaccharides, such as chitin, carboxymethyl cellulose, pectin and starch but is unable to hydrolyze tyrosine (28). The G+C ratio ranges from 29.8 to 42.9 mol% (30).

1.4.2 Bacterial culture and isolation

F. columnare is a thermophilic microorganism that can grow at temperatures ranging from 4°C to 30°C, with optimum growth between 25°C to 30°C (27, 29).

F. columnare colonies are generally yellow-pigmented on solid agar but can vary from being almost colorless or offwhite to creamy or bright orange. On highly nutrient solid agar the colonies are shiny, circular, domed or low domed, with intact or sinuous edges. Compared to other *Flavobacterium* species, *F. columnare* colonies appear flat or very thin on low nutrient solid agar. Furthermore, the colonies can appear as a spreading mass with uneven or filamentous margins that may be adherent on the agar (28).

F. columnare is commonly cultivated using cytophaga agar, as described by Anacker and Ordal (29). Chase, Shieh and Liewes media have also been shown to be successful in culturing this bacterium with shorter incubation time and higher yields, but these media contain salt, which cytophaga agar lacks (30, 31). *F. columnare* can also be grown on modified Shieh broth (32, 33) and can be selectively cultured using Shieh medium containing polymyxin, neomycin or tobramycin (34, 35).

1.4.3 Virulence factors

F. columnare has been isolated from diseased fish, usually from shallow lesions and internal organs (28, 29).

Kleisus *et al*. suggested that fish mucus might act as a chemo attractant for the bacterium. They concluded furthermore that the mucus from the surface of the fish, such as skin and gills is a more effective chemo attractant for *F. columnare* than mucus from the intestine. (36) Later Kleisus *et al.* demonstrated that if *F. columnare* cells were pre-treated with sodium metaperiodate, their chemotactic response to skin mucus was significantly inhibited (32).

A gliding motility gene, *gldH,* has been linked to chemo attraction of *F. columnare* as it shows significant up-regulation when the bacterium is treated with fish skin mucus. However, the up-regulation of *gldH* can be blocked using D-mannose, suggesting that the carbohydrate may work as preventative treatment. Additionally, sodium metaperiodate and D-mannose have been shown to inactivate chemotactic receptors associated with *F. columnare* capsule causing disruption of the capsule (32).

In a study where spontaneous colony morphologies of *F. columnare* were studied, Kunttu *et al.* found that even though colony types had strong adhesion to the agar, the adhesion did not have corresponding affect on virulence and suggested that other factors might play a bigger role in the pathogenesis of *F. columnare* at the start of an infection (33).

Additional bacterial virulence factors have been described, e.g. AC lyase and extracellular proteases, although they do not appear to be sufficient to cause disease (37-39).

1.4.4 Columnaris disease

Columnaris disease has caused major economic loss all around the world. It mainly affects cold and warm freshwater fish and has been isolated from fish such as channel catfish, *Ictalurus punctatus*, Nile tilapia, *Oreochromis niloticus* (L.), common carp, *Cyprinus carpio* L., Indian carp, *Catla catla*, climbing perch, *Anabas testudineus*, striped catfish, *Pangasianodon hypophthalmus* and rainbow trout, *Oncorhynchus mykiss* (40).

1.4.5 Clinical signs

The main characteristics of columnaris disease are lesions on both skin and fins. The gross saddle lesions are iconic for this disease and patches that later become saddle lesions appear to be of a lighter color than the rest of the skin and have smooth edges. In general, the patches can occur all over the body but they appear mostly between the dorsal fin and the caudal fins with the edges extending down both sides towards the lateral line. Patches can fuse together making larger patches and lesions (29). Ajmal and Hobbs described hemorrhagic patches growing at the base of the ventral fins as well as hemorrhagic lesions around the mouth and on the head cartilage of English roach (*Rutilus* rutilus) and perch (*Perca fluviatilis*), as well as pathological changes in liver and kidney. In the study by Ajmal and Hobbs, the gills showed no signs of disease but in a later study by Wolke *et al.,* gill lesions were detected that involved blockage, blotting without any color and massive loss of gill filaments (29, 41).

While *F. columnaris* has been isolated in neighboring countries, the bacterium has not yet been isolated in Iceland (41, 42).

1.5 Tenacibaculum maritimum

Initially, *T. maritimum* was referred to as *Flexibacter maritimus*, based on its morphology and DNA-DNA hybridization pattern. Later it became a part of a new genus, *Tenacibaculum,* and was renamed *Tenacibaculum maritimum* based on the nucleotide sequence of the *gyrB* gene (4, 43, 44).

1.5.1 Morphology and physiological characteristics

T. maritimum is a Gram-negative bacterium with cells growing as flexible slender rods that create gliding or creeping movement without flagella. The rod-shaped cells are 0.1 to 0.5 µm in width and range from 0.1 to 30 µm in length. *T. maritimum* is the only tail and fin rot bacteria discussed here that has been shown to be able to produce fruiting bodies. However, cells in prolonged culture fail to produce fruiting bodies and may become shorter (43, 45).

Biochemical testing of *T. maritimum* strains shows that there is no degradation of agar, chitin, starch or esculin, while the bacterium degrades casein, tributyrin, gelatin and tyrosine. Nitrogenous compounds like tryptone, casamino acids and yeast extract are utilized (43) and several different carbohydrates do not promote the production of acids. Furthermore, the bacterium does not produce ammonium, indole, catalase and some differ in the production of hydrogen sulfide (43, 45, 46).

The G+C content ranges from 31.3 mol% to 32.5 mol% (43).

1.5.2 Bacterial culture and isolation

T. maritimum is a saltwater bacterium that is cultured on specialized, non-selective and low-nutrient media and grows at temperatures ranging between 15°C and 37°C with optimum growth rate at 25° to 30°C and sea water tolerance of 30% to 100% (43, 45-47).

On cytophaga agar prepared with saltwater, *T. maritimum* colonies appear pale yellow or yellow in color, flat and thin with uneven edges (43, 45)

Comparing different types of media, FMM proved to be most effective, with the highest recovery rate at high dilutions (47).

1.5.3 Virulence factors

Iron is an essential factor for persistence of a pathogen within the host. In general, when a pathogen has found itself within an animal it acquires all of its nutrients from the host tissue. Iron is, however, difficult for bacteria to obtain, as it is not freely available within tissues and its concentration very low. Consequently, pathogens use special mechanisms for the uptake of iron from the host (48). Avendaño-Herrera *et al*. reported that *T. maritimum* possesses at least two distinct systems for the uptake of iron; a system involving the synthesis of siderophores and another system involving utilization of heme groups as an iron source by direct binding (49).

T. maritimum secretes extracellular products (ECP) that are known to be important for degrading host tissue. The ECP produced by *T. maritimum* are highly toxic and can cause cellular necrosis in internal organs (50).

1.5.4 Marine Tenacibaculosis

T. maritimum was described as an agent of fish disease in Japan in 1977 (4). However, the economic impact of the bacterium did not become fully clear until it caused mass mortality in Dover sole (*Solea solea*) in Scotland in 1979 (51). Since then, major outbreaks of the disease, marine tenacibaculosis, have occurred in Spain, Portugal, France and Italy. Furthermore, smaller outbreaks have been reported in locations such as Australia and North America (4).

1.5.5 Clinical signs

The most common clinical signs of marine tenacibaculosis are body lesions that include areas of scale loss, as well as ulcers extending into the muscular tissue. Other noticeable symptoms include lesions on the head, eyes, fins and gills (52). Post-smolts are particularly susceptible to marine tenacibaculosis and can show hemorrhaging of abdominal breast, swelling and destruction of buccal

cavity and jaws, hyperemia of meninges, tissue around the orbital cavity and lower intestine and bloody ascites (53).

The onset of marine tenacibaculosis is characterized by a darkening of the skin between caudal and marginal fin rays, followed by slight blistering of the skin. Subsequently, these darker patches get bigger and the epithelial surface starts peeling off and the underlying tissue gets exposed with hemorrhaging (51). Hemorrhaging and frayed fins can be seen at early stages, while dermal lesions become more dominant at later stages of disease. The bacteria may become invasive, transmitting to the epidermis, dermis and muscular layers (45, 53).

1.6 Tenacibaculum soleae

1.6.1 Morphology and physiological characteristics

T. solea is a Gram-negative bacterium. The colonies consist of rods, ranging from 2 to 25 µm in length and a diameter of 0.5 µm (3, 54-56).

By staining with Congo red stain, carotenoid pigments can be detected. The bacterium hydrolyses gelatin, casein and DNA but does not hydrolyse esculin, starch or tween 20 and 80. Enzymes produced are alkaline phosphatase, esterase, leucine arylamidase and valine arylamidase. It does not produce trypsin, α-chemotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase or α-fucosidase but some strains differ in terms of a production of esterase lipase, lipase, cystine arylamidase, acid phosphatase, phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase (3, 54-57).

The DNA G+C content has been estimated to be 29.8 mol% (3).

1.6.2 Bacterial culture and isolation

T. soleae is a thermophilic marine bacterium which grows at temperatures ranging from 15°C to 30°C, with optimal growth ranging from 22°C to 25°C, and sea water tolerance of 55% to 100%, therefore not growing in freshwater (3, 56). It grows as yellow or bright yellow colonies with spreading and uneven margins on FMM and Marine agar (MA). Additionally, the bacterium has gliding motility and does not adhere to agar plates (3, 54-57).

T. soleae is sensitive to florfenicol, novobiocin and sulfamethoxazole-trimethoprim, showing average response to enrofloxacin, erythromycin and flumequine and resistance to oxytetracycline, ampicillin and doxycycline (55, 56).

1.6.3 Virulence factors

The virulence of *T. soleae* is comparable to the virulence of *T. maritimum.* A challenge study using *T. soleae* isolate a47 showed 100% mortality within 6 to 8 days when fish were infected using bath immersion or intraperitoneal injection (56). When *T. soleae* isolate TS21-10 was used, no mortality was observed when fish were infected intraperitoneally, but fish infected by bath immersion showed 60% mortality 8 days post-infection (55, 57). In this transmission study, both virulent strains, a47 and TS21-10, could be isolated from internal organs, indicating a systemic infection. These results indicate

that the disease can be easily propagated by bath challenge, even without scraping fish skin prior to challenge (55-57).

In both of these studies, a serological characterization revealed that *T. soleae* strains only reacted with antiserum when “O” antigen from homologous strains was used, which underscores antigenic variability among *T. soleae* strains (55-57).

1.6.4 Tenacibaculosis

T. soleae, the causative agent of the disease tenacibaculosis, was only recently discovered (2008) but can cause significant mortality worldwide in commercially valuable species such as Senegalese sole (*Solea senegalensis*), wedge sole (*Dicologoglossa cunneata*) and brill (*Scophthalmus rhombus*) (3, 56).

1.6.5 Clinical signs

Infection outbreaks have been reported in various age groups, i.e. fry, juvenile and adult stages of wedge sole, adult brill and juvenile and adults of Senegalese sole. The main clinical signs are ulcers on the body and tail. Furthermore, some fish show other symptoms, such as petechial hemorrhaging of fins, jaws and the ventral body, anemia and wandering swimming behavior (54, 56).

1.7 Tail and fin rot in Icelandic aquaculture, treatment and preventive actions

Aquaculture is an expanding industry. Over 160 fish species were believed to be cultured in 2012 and world production has increased by tenfold from 1970 (58). Aquaculture started in Iceland in 1951 and today the main cultured species are Artic charr and Atlantic salmon (59).

Flavobacterium sp. is first mentioned in Iceland in the annual report of fish disease in 2007 where the bacterium was described as an opportunist in Icelandic aquaculture. In 2012 five disease outbreaks in fresh water aquaculture of both Artic charr and rainbow trout, were linked to *F. psychrophilum* by using phenotypical examination (60, 61).

Antibiotics are often used to treat *F. psychrophilum, F. columnare* and *T. maritimum* infections causing tail and fin rot as well as chemical treatments like potassium permanganate and salt and acid bath treatments (4, 25, 27, 62-64). Antibiotics can be effective treatment for BCWD, columnaris disease and marine tenacibaculosis but publications concerning the treatment of tenacibaculosis were not found. Although antibiotics are effective for treatment, bacterial development of antibiotic resistance is a major challenge. Resistance to oxytetracycline, amoxicillin and oxolinic acid has already been described for *F. psychrophilum* (65-67) and *F. columanare* (68) and colistin, kanamicyn, neomicyn, oxolinic acid and flumequine for *T. maritimum* (4). Florfenicol is still used effectively for treating *F. psychrophilum* (67) *F. columnare* (69) and *T. soleae* (55) and enrofloxacin for *F. maritimum* (4).

Vaccines are considered a part of general fish health management to prevent diseases. Several types of vaccines for *F. psychrophilum* have been tested, including live attenuated and live non-

attenuated strains (70-72), formalin-killed bacteria (73), heat-inactivated bacteria (74) and vaccines using specific *F. psychrophilum* antigens (75). None of the vaccines, however, are commercially available yet (25).

A vaccine containing live attenuated bacteria is commercially available in the USA for the prevention of columnaris disease with relative percent survival up to 94% in channel catfish and largemouth bass fry (76). In addition, immunization using formalin-killed bacteria resulted in significant systemic humoral responses, with a threefold increase in antibody levels in tilapia (77), and immersion of channel catfish resulted in a significant decrease in columnaris disease compared to unvaccinated fish (78).

A bacterin vaccine is currently available for *T. maritimum* in turbot (79) and there is an ongoing research in Spain to develop a *T. maritimum* bacterin vaccine specific for cultured sole (80). Publications concerning the prevention of tenacibaculosis using vaccines were not found.

No matter which the causative agent is, disease management is crucial. Mortality can be reduced with regular feeding of a nutritious diet, and minimizing physical handling and stress of the fish. In addition, prompt removal of dead and diseased fish can help reduce disease transmission by reducing the infectious load in the environment (62, 81).

1.8 Pathogen identification

In addition to traditional methods of identifying fish pathogens, such as serology, histology and bacterial morphology, newer methods are gradually being incorporated. These methods are based on molecular biology and are faster and potentially more sensitive than traditional methods and can be used to detect genetic variation of subspecies or strains.

The importance of molecular biology is reflected in examples of phenotypically homologous characteristics within *F. columnare* strains isolated from various hosts in different geographical areas that were reclassified based on nucleic acid sequencing (82, 83).

However, molecular methods often require some basic knowledge of the pathogen, and thus traditional methods are still a valuable tool when studying new pathogens, epidemiology, pathophysiology and treatment responses of new diseases. Traditional methods are for example cultivation, Gram-stain reaction, sugar-fermentation prodiles, enzymic activities and indirect fluorescent antibody technique (IFAT) (84). Additionally, culture has the importance of being used in studying clinical and biological features of pathogenic bacteria as well as being important in genetic sequencing (85).

Molecular analyses that have been used for identifying fish pathogens and epidemiological studies include ribotyping, restriction fragment length polymorphism (RFLP), DNA array-based multiplex assay, pulsed-field gel electrophoresis (PFGE) and MALDI-TOF mass spectrometry, but MALDI-TOF mass spectrometry is considered a rapid and low-cost identification method that can reflect the taxonomy derived from the 16S rRNA gene.

Amplification methods are for example random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) fingerprinting, single strand conformation

polymorphism (SSCP), restriction fragment length polymorphism of the 16S rRNA gene (16S-RFLP), PCR amplification of the 16S rRNA gene, the 16S – 23S IGS region, plasmid DNA and DNA gyrase and multilocus sequence typing (MLST) (26, 82, 86-98).

MLST is a high-resolution characterization of isolates based on the alleles of seven “MLST loci” or housekeeping genes. The sequence for each loci receives an allele number that is combined into an allelic profile or a sequence type. The allelic profile or sequence type can then be used to define strains. Each allelic profile represents thousands of base pairs of information and consequently many hundreds of alleles at each locus and thousands of sequence types have been identified for bacteria examined with MLST. The advantages of MLST are that the results can be compared to results from other laboratories and are easily interpreted. Some of the disadvantages of this method are that the variability of housekeeping genes makes it difficult to compare anything but closely related bacteria and that the MLST method cannot provide detailed discrimination for low diversity and single clone pathogens (99).

1.8.1 Pathogen identification using PCR

PCR uses short nucleic acid sequences, called primers, to bind DNA and amplify the target sequence between the primers. The amplification is done with a thermocycling reaction where the DNA template is denatured, primers anneal to their target regions and a DNA polymerase enzyme creates new copies of the desired DNA region in each round. The PCR product can later be analyzed using electrophoresis and sequencing (81).

The 16S rRNA gene is well conserved within all bacteria and is believed to serve a critical cellular function (100). The 16S rRNA gene consists of both variable and conserved regions and is therefore useful for species-specific primer pair design and species identification when working with an unknown organism. Sequencing the 5’ third of the 16S rRNA gene has generally been considered to provide enough taxonomic information for identification but genetic comparison of the entire 16S rRNA gene (about 1500 bp) is required when describing a new species (101, 102). However, the usefulness of 16S rRNA gene comparison is limited in species with highly similar sequences. Thus, for comparing sequences within genera comparison, genes other than 16S rRNA may be useful (101, 103).

Species-specific primer pairs, targeting the 16S rRNA gene, have been designed for the detection of *F. psychrophilum, F. columnare*, *T. maritimum*, *T. soleae* and *F. branchiophilum*; FP1–FP2 (104) and PSY1–PSY2 (105) for *F. psychrophilum,* Col1–Col2 (106, 107) and ColF–ColR (108) for *F. columnare,* MAR1–MAR2 and Mar1–Mar2 (107) for *T. maritimum,* Sol-Fw–Sol-Rv (109) for *T. soleae* and BRA1–BRA2 for *F. branchiophilum*. Most of the PCR primers target the 16S rRNA gene, where universal primers first amplify the 16S rRNA gene, followed by nested PCR using species-specific primers situated within the 16S rRNA gene.

The intergenic spacer region (IGS) between the 16S and 23S rRNA genes and the *gyrB* gene have also been used as target regions for species-specific primers. The 16S – 23S IGS region is a stable and conserved area and is believed to be under less evolutionary pressure than the 16S rRNA gene and can therefore provide a greater genetic diversity than the 16S rRNA gene (110).

Primers targeting the *gyrB* gene for *F. psychrophilum* (PSY-G1F and PSY-G1R) (91) and primers targeting the 16S-23S IGS region for *F. columnare* (FCISRFL–RCISRR1) (111) and for *T. soleae* (G47F–G47R) (112) have been described.

Flavobacterium and *Tenacibaculum* are closely related bacteria, based e.g. on the sequence similarity of the 16S rRNA gene (101). It is therefore important to include *Flavobacterium* and *Tenacibaculum* bacteria, especially the ones that are considered fish pathogens, when species-specific primers are being designed, to prevent species-non-specific amplification. However, the availability of 16S rRNA gene sequences from related species varies between species (104-109, 111, 112). Several attempts have been made to design species-specific 16S rRNA gene primers for *Flavobacterium* and *Tenacibaculum* species. However, the quality of the primers has largely been insufficient due to species-non-specific amplification (113, 114), hairpin formation, primer-dimer formation, intra-specific genomic variation and poor sensitivity (115, 116).

By optimizing PCR amplification conditions, e.g. by increasing the annealing temperature, a better amplification can be obtained and species-non-specific binding decreased (104, 107, 113).

To obtain better detection sensitivity nested PCR approach is widely used (116). A touchdown approach can be used to optimize PCR reactions with increasing sensitivity, specificity and yield. In touchdown PCR, the initial annealing temperature is higher than the projected annealing temperature of the primers being used (117). The high initial annealing temperature produces a specific amplification of low yield. By gradually lowering the annealing temperature to a more permissive temperature, the initial product serves as a specific template that will out-compete other possible target sequences (118).

Rapid and accurate identification of etiological agents of disease are important for treatment of disease and vaccine development. It is also important for epidemiological studies and to understand host specificity and pathogenicity (42).

1.9 Heat and salinity, influence on growth

Recently publications focusing on the effects of water temperature and salinity on the virulence of *F. psychrophilum* showed that the bacterium is adapted to cold freshwater. The bacteria grew better at 15°C than 5°C and 10°C, and better in fresh water than in brackish and artificial salt water(119). Although the best growth rate is at 15°C, *F. psychrophilum* is considered a cold water species because it can cause disease symptoms at 4 – 10°C, which are considered low temperatures (119). Low metabolic activity is believed to explain the limited bacterial growth at 5°C (119). Bacteria cultured in brackish water reduced in cell count. However, bacteria could be recovered, which indicates even though brackish water is detrimental to the bacteria, some *F. psychrophilum* may be albe to survive. No viable bacteria cells were detected after seven days in saltwater culture, which indicates that it is highly unlikely that *F. psychrophilum* will survive in marine water (119).

These temperature and salinity tolerance tests were reproduced with bacterial fin and tail rot isolates from Iceland, in order to study the optimal growth conditions for Icelandic *Flavobacterium* sp.

2 Aim

This thesis is a part of a research project sponsored by AVS (AVS R&D Fund, Ministry of Fisheries and Agriculture in Iceland) that aims to understand the distribution and variability of bacteria causing tail and fin rot in Iceland, as well as studying bacterial transmission and development of vaccines to prevent tail and fin rot in aquaculture.

The aim of this project was to isolate bacteria causing tail and fin rot disease in wild and farmed Icelandic fish, to develop methods for identification of the bacteria, and to determine the optimal growth conditions of selected isolates. Genetic analysis was performed to determine the variability between bacterial isolates. The goal of this project was to identify the causative agents of tail and fin rot in Iceland, and to explore the likelihood of the pathogens crossing environmental barriers.

The specific aims of the project can be divided into three parts:

1. Identification of Icelandic *Flavobacterium* and *Tenacibaculum* species using published species-specific PCR primers and construction of new species-specific PCR primers for *F. psychrophilum, F. columnare, T. maritimum*, *T. soleae* and *F. branchiophilum*

2. Construction of phylogenetic trees to describe relationships among species

3. Measuring bacterial growth of *F. psychrophilum, T. maritimum* and *T. soleae* reference strains and Icelandic field strains under different salinity and temperature conditions

3 Materials and Methods

3.1 Sample collection

A total of 211 bacterial isolates (blue) were obtained from fish and water samples during the years 2014 and 2015, Fish samples (green) included both farmed fish (light green) and wild fish (light blue) in Iceland, living either in freshwater or seawater (grey) and roe samples included both unfertilized and fertilized roe (yellow). Water samples included intake water from fish farms, as well as roe water (83) (Figure 2).

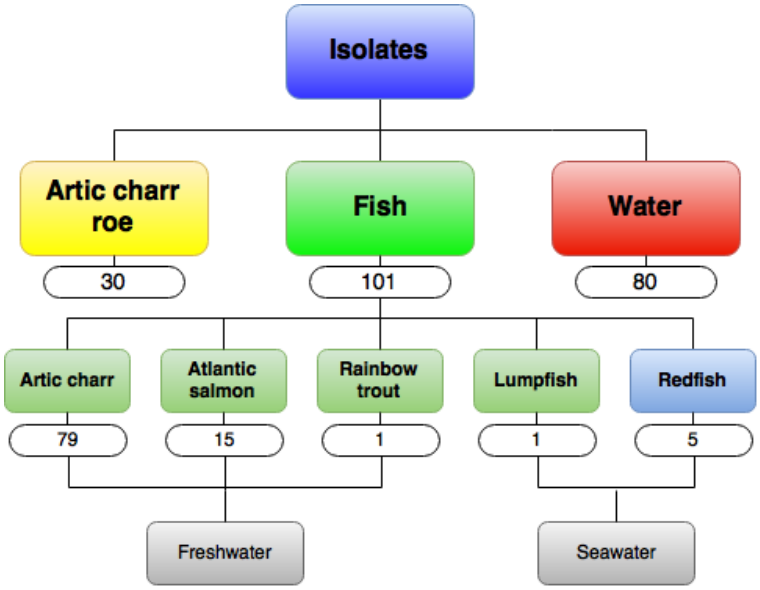


Figure 2. Number of bacterial isolates and their origin. Collection of isolates (bright-blue) fall into three categories: Artic charr roe samples (yellow), fish samples (bright-green) and water samples. Isolates cultured from fish are divided into farmed fish (green) and wild fish (blue) living in freshwater or seawater (grey).

3.1.1 Farmed fish

Most fish farms in Iceland were offered to take part in this project. Fish were either sent to Keldur for sampling, or samples collected on-site at the fish farms. Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*) were farmed in freshwater while lumpfish (*Cylopterus lumpus*) and rainbow trout (*Oncorhynchus mykiss*) were farmed in seawater. Samples from wounds and/or healed lesions from fish were pooled together, ground in a mortar and inoculated on FMM agar (Appendix I). Samples taken from farmed freshwater fish were diluted from ten up to million times with phosphate-buffered saline (PBS, Appendix I) before plating on FMM agar without salt while samples from seawater fish were diluted with minimal salt solution (MSS, Appendix I) and inoculated on FMM agar containing seawater.

3.1.2 Wild fish

A trip to Ísafjörður was made in September 2014 to collect wild fish at sea. Both Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) were sampled. Samples were collected from

symptomatic fish where lesions and healed lesions were scraped and pooled. Scraped samples were ground in a mortar with MSS, diluted with MSS as before and inoculated on FMM agar containing seawater.

Samples of redfish (*Sebastes marinus*) retrieved from a fishmarket were ground and cultured the same way as for the wild fish.

3.2 *Flavobacterium* and *Tenacibaculum* isolation

3.2.1 Bacterial culture

After spreading 100 µL of diluted samples on FMM agar plates with a glass rod the agar plates were incubated at 15°C for 5 to 7 days, or until yellowish colonies could be visualized. In most cases, a mixed bacterial culture grew. To acquire pure culture, yellow colonies were re-streaked on new agar plates until pure growth was obtained.

3.2.2 Microscopic examination

We used a light microscope (SM-Lux, Leitz Wetzlar) in order to screen for bacterial cells typical for tail and fin rot, i.e. long and slender Gram negative bacteria. Wound scrapings from fish were placed directly on a microscope slide with a drop of PBS, covered with a coverslip and examined. Gram stained slides with bacterial growth were also examined.

3.3 Isolation of DNA

DNA extraction was done on pure cultures from agar plates using NucleoSpin® Tissue kit (Macherey-Nagel, catalog nr. 740952). Approximately one 10 µL loopful of culture was added to 200 µL of lysis buffer T1 and treated with 25 µL Proteinase K (Macherey-Nagel) and buffer B3. The solution was then incubated at 56°C for 1 to 2 days, or until the samples were completely lysed. Ethanol was added to the solution and the samples centrifuged through a column in a collection tube after mixing. The sample was washed with wash buffers (wash buffer BW and B5) and then centrifuged without any solution to dry the silica membrane. The column was placed into a 1.5 mL tube and a pre-warmed (70°C) 5mM Tris/HCl, pH 8.5 elution buffer BE added. After 1 to 3 minutes, the column was centrifuged. The eluted DNA was then analyzed for quality and quantity in a NanoDrop ND-1000 spectrophotometer (according to the manufacturer's directions) and stored at -20°C until use.

3.4 DNA amplification using 16S rRNA species-specific primers

Previously published species-specific primers were used for identification of *F. psychrophilum*, *T. maritimum* and *T. soleae.* They are all displayed in Table 1 and their target sequences are listed in Appendix II.

Table 1. 16S rRNA gene species-specific primer pairs used for the detection of different tail and fin rot bacteria.

Target bacteria	Forward primer	Reverse primer	Product size (bp)	Reference
F. psychrophilum	PSY1	PSY2	1089	(105)
T. maritimum	MAR1	MAR2	400	(114)
T. soleae	Sol-Fw	Sol-Rv	248	(109)

3.5 Primer design

Species-specific primers were designed for *F. psychrophilum*, *F. columnare*, *T. maritimum*, *T. soleae* and *F. branchiophilum* from the 16S rRNA gene of the bacterial genome and the 16S – 23S IGS region using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Sequences were downloaded from the NCBI gene database (https://www.ncbi.nlm.nih.gov/pubmed/) and analyzed using Sequencher (v. 4.8) (Gene Codes Corporation). In Table 2 strain numbers are shown for each sequence used for the construction of new primers. All primers were purchased from TAG Copenhagen and are listed in Appendix II. Primers were tested using genomic DNA from *F. psychrophilum*, *F. columnare*, *T. maritimum*, *T. soleae* and *F. branchiophilum* strains, as well as isolates collected in this study (Table 3).

Table 2. Bacterial strains used for constructing new primers.
Strain number is shown for each bacterial species. Bold: reference strains

16S rRNA and Intergenic spacer (IGS)	
Bacterial species	**Strains**
F. psychrophilum	**NCIMB 1947**, IFO 15942, Fp-C, FPC814, 1779, MH1
T. soleae	a410, LL04 12.1.7, a216, a47
T. maritimum	**ATCC 43398**, NUF1128, JCM 8137, NBRC 15946
F. columnare	CUVET1215, Ga-6-93
F. branchiophilum	FL-15

Table 3. Bacterial strains and isolates used to test the specificity of new primers.
Bold: Strain numbers are shown for the reference strains, purchased from The Global Bioresource Center (ATCC). Strains in *italic* were generously provided by the Department of Molecular and Cellular Biology at the University of Guelph. Other isolates were collected in this project.

Bacterial species	Strains/Isolates
F. psychrophilum	**NCIMB 1947**, 140. 18, 82, 9, 113, 129, 132
T. soleae	**NCIMB 14368**
T. maritimum	**ATCC 43398**
F. columnare	*RSFL151*
F. branchiophilum	*RSFL136,* **ATCC 35035**
Pseudomonas sp.	94
Vibrio sp.	172

3.6 PCR

PCR reactions were performed on a Thermal Cycler 2720 from Applied Biosystems and Veriti 96 Well Thermal Cycler from Applied Biosystems. GE Healthcare Illustra^{TM} PuReTaq Ready-To-Go PCR beads or New England BioLabs *Taq* 2X Master Mix were used for the amplification reactions.

3.6.1 PCR reaction solution using Illustra PuReTaq Ready-To-Go PCR beads.

10µM of primers, forward and reverse and 22 µL of ddH2O were added to PCR solution as well as 150 to 350 ng DNA that was used as a template in primary PCR reactions. The product from the first PCR was diluted 10-fold for use in nested PCR reactions.

3.6.2 PCR reaction solution using New England BioLabs *Taq* 2X Master Mix

10µM of primers forward and reverse and 12.5 µL of *Taq* 2X Master Mix were added to PCR reaction as well as 150 to 350 ng template DNA that was used as a template in primary PCR reactions. ddH_2O was added until solution reached 25 µL. The product from the first PCR was diluted 10-fold for use in nested PCR reactions. When $MgCl_2$ was added to PCR solution, the same amount of ddH_2O was replaced.

3.6.3 PCR protocols

PCR protocol 1

1. Denaturation	95°C	5:00 min
2. Denaturation	95°C	0:45 min
3. Annealing	55°C	0:45 min
4. Elongation	72°C	1:00 min
5. Elongation	72°C	5:00 min

Cycle (steps 2 to 4) was repeated 35 times

PCR program 1 was used for universal primers 8F (120), 805R (121) and 1544R (122) as well as PSY1 – PSY2 (105), MAR1 – MAR2 (106), Sol-Fw – Sol-Rv (109), ColF – ColR (108) primers (target sequences are presented in Appendix II).

PCR protocol 2

1. Denaturation	94°C	5:00 min
2. Denaturation	94°C	1:00 min
3. Annealing	57°C*	0:45 min
4. Elongation	72°C	1:00 min
5. Elongation	72°C	5:00 min

Cycle (steps 2 to 4) was repeated 45 times

PCR program 2 was used for primers G47F – G47R (112) (target sequences are presented in Appendix II). *Aiming to reach the maximum specificity for these primers, a PCR program with various annealing temperatures was used (touchdown PCR) (123).

PCR protocol 3

1. Denaturation	94°C	5:00 min
2. Denaturation	94°C	1:00 min
3. Annealing	56°C	1:00 min
4. Elongation	72°C	2:00 min
5. Elongation	72°C	5:00 min

Cycle (steps 2 to 4) was repeated 35 times

PCR program 2 was used for primers GYR-1 – GYR-1R and PSY-G1F – PSY-G1R (91) (target sequences are presented in Appendix II).

PCR protocol 4

1. Denaturation	95°C	5 min
2. Denaturation	95°C	30 sec
3. Annealing	Tm°C*	30 sec
4. Elongation	72°C	1 min
5. Elongation	72°C	7 min

Annealing temperature started at 5°C above the described Tm for each primer pair, was decreased by 0.5°C per cycle (steps 2 to 4) for 14 cycles and then carried out for 25 more cycles without lowering the temperature.

PCR program 4 was used for primers PsyF, PsyR, Psy-3F, Psy-3R, Psy-4F, Psy-4R, Psy-5F, Psy-5R, MarF, MarR, Mar-1F, Mar-1R, Mar-2R, SolF, SolR, ColF, ColR, Col-1F, Col-1R, Col-2R, BraF and BraR (target sequences are presented in Appendix II). Aiming to reach the maximum specificity for these primers, gradient PCR was used, i.e. touchdown PCR.

3.7 DNA electrophoresis

PCR products were run on 0.8 to 2.0% agarose gels. Agarose Basic (AppliChem) powder was melted in 0.5x TBE (Tris borate-EDTA, see Appendix I) and ethidium bromide (0.13 ng/mL) added before the solution solidified. Before loading PCR products on the gel, 10x RSB (Restriction buffer, see Appendix I) was added to the sample. Electrophoresis was performed at 70V for 50 to 65 min, depending on the size of DNA fragments. To estimate the size of fragments, 2-log ladder (New England Biolabs) was loaded to the gel as well. Finally, PCR products were visualized under UV light in InGenius (SynGene) and imaged using the GeneSnap program (SynGene).

3.8 Extraction of DNA from agarose gel and DNA quantification

3.8.1 PCR clean-up Gel extraction kit

NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, catalog nr. K0692) was used for gel extraction of PCR products. All centrifugation was performed at 11000 x g for 1 minute. Thirty seconds were added to the time for a complete filtering through the matrix on the column. The DNA was visualized under UV light and excised from the gel. The weight of the gel pieces containing the DNA fragments was determined and 200 µL of Binding buffer NT1 was added to each 100 mg of gel piece. Samples were incubated for 10 minutes at 50°C or until completely dissolved. Samples were loaded onto a column in a collection tube and centrifuged. The samples were then washed twice with 700 µL of Wash buffer NT3 and then centrifuged without any solution to dry the silica membrane. The columns were placed into a 1.5 mL tube, 30 µL of 5 mM Tris/HCl, pH 8.5 (Elution buffer NE) was added and incubated at room temperature for 1 to 3 minutes before centrifugation. The products were then analyzed for quality and quantity in a NanoDrop spectrophotometer and stored at -20°C until use.

3.8.2 GeneJET Gel extraction kit

GeneJET Gel extraction kit (Thermo Scientific, catalog nr. 740609) was used for gel extraction of PCR products. DNA was viewed under UV light and excised from the gel. A total of 400 µL of binding buffer was added to all gel fragments containing DNA and the samples incubated for 10 minutes at 50°C or until completely dissolved. Samples were loaded onto a column in a collection tube and centrifuged. All centrifugation was at 11000 x g for 1 minute. Samples were washed twice with 700 µL of wash buffer and centrifuged without any solution to dry the silica membrane. The columns were placed into a 1.5 mL tube, 30 µL 10 mM Tris-HCl, pH 8.5 (Elution buffer) was added and incubated at room temperature for 1 to 3 minutes before centrifuged. The product was then analyzed for quality and quantity in a Nanodrop spectophotometer and stored at -20°C until use.

3.9 Sanger sequencing

The 16S rRNA gene was amplified with universal primers 8F and 1544R. PCR product was purified and sequenced using the same primers in addition to the Primer 805R, when needed. Samples were sent abroad to Macrogen or Beckman Coulter for Sanger sequencing using ABI 3730XL sequencer. AB1 Formatted DNA sequences were analyzed using Sequencher (v. 4.8) (Gene Codes).

3.10 Phylogenetic analysis

Nucleotide sequences used were both from this study (211 isolates) and retrieved from the NCBI database (3 reference strains). The phylogenetic analysis is composed of 16S rRNA sequences from isolates that were obtained from culture and gave sufficiently long sequencing reads. Sequences of interest were aligned using ClustalX (v. 2.1) program (124). Alignment files obtained from ClustalX were imported into MEGA (v. 7), (125). Sequences were aligned and all gaps and inconclusive bases were eliminated from the analysis with MUSCLE alignment (126) using UPGMB clustering method. Aligned sequences were saved in .meg format and used for phylogenetic tree constructions. Five types of phylogenetic trees were constructed with 500 bootstrap replications; maximum-likelihood tree, neighbor-joining tree, minimum-evolution tree, UPGMA tree and maximum-parsimony tree. Settings used when constructing phylogenetic trees are listed in Table 8 (Appendix IV).

3.11 Measurement of bacterial growth

Bacteria of interest were sub-cultured on FMM agar to the point where one inoculation loop fill (10 µL) of bacteria could be inoculated in a 15 mL tube (Sarstedt) containing 6 mL of liquid FMM medium (Appendix I) and vortexed vigorously. After 4 days of cultivation under experimental conditions, 250 µL of liquid culture were inoculated in three prepared 15 mL tubes containing 6 mL of liquid FMM medium. Bacterial density was measured in units of optical density (OD) by a GeneQuant *pro* spectrophotometer (Amersham Pharmacia Biotech) at 600 nm. Two experimental variables were examined; salinity and temperature.

Prior to day 0, primary culture for each bacterium was sub-cultured for three days. Each experiment was carried out for 5 days and measurements were performed at days 0, 1, 2, 3 and 4.

The measurements were done with three parallel cultures for each treatment. OD values of 45 mm cuvettes (Sarstedt) containing 1500 µL of culture were measured. Results were calculated and plotted using GraphPad Prism (v. 6) (GraphPad Software). FMM of different ppm salinity levels, for testing salinity tolerance of the bacteria (full salinity: 32 ppm), is displayed in Table 4 as well as temperature levels, used for testing temperature tolerance. GraphPad Prism was used for statistical analysis (two-factor ANOVA) with $p<0.05$ set as the critical value of significance.

Table 4. Experimental conditions testing tolerance of salinity and temperature.

Salinity tolerance experiment 1 and 2		**Temperature tolerance experiment 1 and 2**	
Bacteria	**Salinity (ppm)**	**Bacteria**	**Temperature (°C)**
F. psychrophilum	0	***F. psychrophilum***	5
	16		10
	32		15
T. maritimum	0	***T. maritimum***	5
	16		10
	32		15
T. soleae	0	***T. soleae***	5
	16		10
	32		15

4 Results

4.1 Identification of tail and fin rot bacteria using nested 16S rRNA gene PCR.

The 16S rRNA gene is 1541 bp in length (127). When it is amplified with universal primers 8F and 1544R, a PCR product of 1536 bp is created (Figure 3A). Species-specific primers are situated within this part of the 16S rRNA gene and their product sizes are listed in Table 1 and shown in Figures 3B – D. The universal 16S primers and species-specific primers were used with PCR protocol 1 (see section 3.6.3).

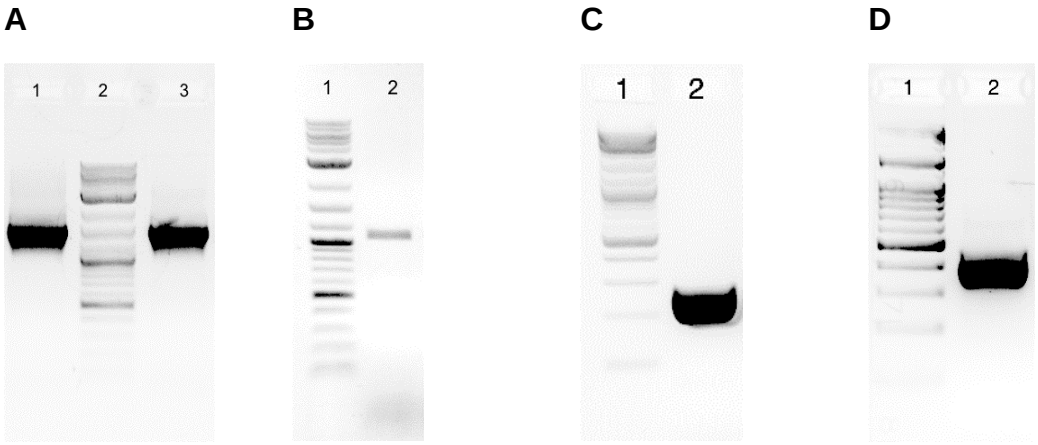


Figure 3. 16S universal primers and 16S species-specific primers. A: Primers 8F-1544R Lanes: 1 = *T. soleae*, 2 = 2-log ladder, 3 = *T. maritimum*. B: Primers PSY1 – PSY2 Lanes: 1 2-log ladder, 2 = *F. psychrophilum*. C: Primers Sol-Fw – Sol-Rv Lanes: 1 2-log ladder, 2 = *T. soleae*. D: Primers MAR1 – MAR2 Lanes: 1 2-log ladder, 2 = *T. maritimum*

When reference sequences and sample isolates were amplified with published species-specific primers (Table 1) some species-non-specific amplification was observed. Examples of this species-non-specific amplification are shown in Figure 4 (lanes with an asterisk). Irrelevant lanes were trimmed out in Figure 4B.

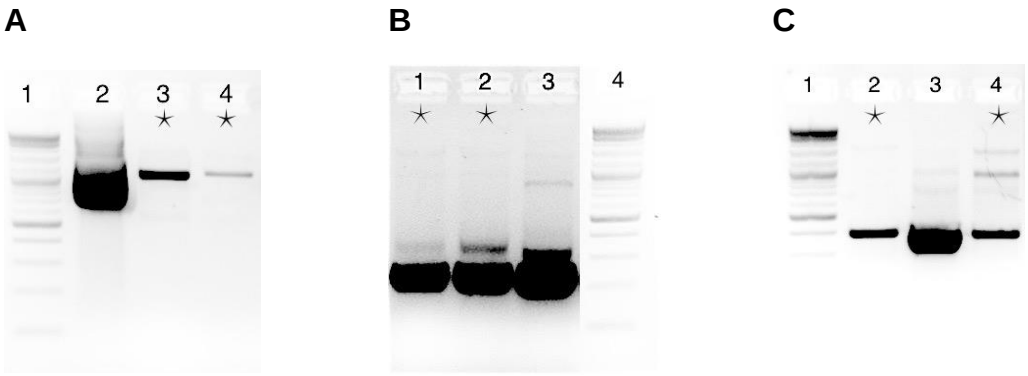


Figure 4. Species-non-specific amplification using 16S species-specific primers.
A: Primers PSY1 – PSY2 Lanes: 1 2-log ladder, 2 *F. psychrophilum*, 3 *T. maritimum*, 4 *T. soleae*, B: Primers Sol-Fw – Sol-Rv Lanes: 1 *F. psychrophilum*, 2 *T. maritimum*, 3 *T. soleae*, 4 2-log ladder. C: Primers MAR1 – MAR2 Lanes: 1 2-log ladder 2 *F. psychrophilum*, 3 *T. maritimum*, 4 *T. soleae*. Lanes marked with an asterisk indicate species-non-specific amplification.

4.2 Identification of tail and fin rot bacteria using new PCR primers

Due to species-non-specific amplification results with previously described primers (Figure 4), the 16S rRNA gene from Icelandic isolates was sequenced in order to develop new PCR primers with improved specificity. 16S rRNA gene sequencing revealed species-specific sequences between related species, which suggested that more specific primers could be developed.

4.2.1 16S rRNA gene primers

Published 16S rRNA gene sequences were compared to 16S rRNA gene sequences from Icelandic isolates to find regions that were variable between bacterial species and conserved within each species. Several regions within the 16S rRNA genes appeared to be distinctive enough to design species-specific PCR primers. One primer pair targeting the 16S rRNA region was made for each bacterium of interest. The target DNA was amplified using 16S rRNA primer pairs with various annealing temperatures (PCR protocol 4), targeting three reference strains; *F. psychrophilum, T. maritimum* and *T. soleae*. Primer pairs 2, 4 and 5 were used to amplify DNA sequences with strains of *F. branchiophilum* and *F. columnare* as well (Table 5).

When target sequences were amplified with primer pairs 1, 4 and 5 some species-non-specific amplification was recorded, see dark grey boxes and when target sequences were amplified with primer pairs 2 and 5 (Table 5) no amplification was recorded. The target sequences of all primers are shown in Appendix II.

Table 5. List of primer pairs constructed in this study targeting the 16S rRNA gene and PCR amplification results. Tm = Optimal melting temperature of primers. + = amplification of product, - = no amplification of product. PSY = *F. psychrophilum*, MAR = *T. maritimum*, SOL = *T. soleae*, BRA = *F. branchiophilum*, COL = *F. columnare*, NC = negative control, N/D = not done.

						Initial PCR amplification temperature (°C)														
						59°C					61°C					63°C				
Target bacteria	Primer pair	Forward primer	Reverse Primer	Product size (bp)	°Tm (°C)	PSY	MAR	SOL	BRA	COL	PSY	MAR	SOL	BRA	COL	PSY	MAR	SOL	BRA	COL
F. psychrophilum	1	PSYF	PSYR	671	60	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
F. columnare	2	ColF	ColR	846	59	-	-	-	-	-	-	-	-	-	-	N/D	N/D	N/D	N/D	N/D
T. maritimum	3	MarF	MarR	701	59	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
T. soleae	4	SolF	SolR	634	59	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
F. branchiophilum	5	BraF	BraR	297	61	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

	Initial PCR amplification temperature (°C)																	
	68°C						69°C						70°C					
Target bacteria	PSY	MAR	SOL	BRA	COL	NC	PSY	MAR	SOL	BRA	COL	NC	PSY	MAR	SOL	BRA	COL	NC
F. psychrophilum	N/D	N/D	N/D	N/D	N/D	N/D	+	+	+	N/D	N/D	N/D	+	+	+	N/D	N/D	N/D
F. columnare	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
T. maritimum	N/D	N/D	N/D	N/D	N/D	N/D	-	-	-	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
T. soleae	-	+	+	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-
F. branchiophilum	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D

	Initial PCR amplification temperature (°C)								
	71°C						72°C		
Target bacteria	PSY	MAR	SOL	BRA	COL	NC	PSY	MAR	SOL
F. psychrophilum	+	+	+	N/D	N/D	N/D	+	+	+
F. columnare	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
T. maritimum	-	-	-	N/D	N/D	N/D	N/D	N/D	N/D
T. soleae	-	-	-	-	-	-	N/D	N/D	N/D
F. branchiophilum	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D

4.2.2 16S – 23S IGS region primers

Due to species-non-specific amplification with the new 16S rRNA gene primers, 16S – 23S IGS regions of *F. psychrophilum, F. columnare, T. maritimum, T. soleae* and *F. branchiophilum* were aligned in MEGA (v.7). The 16S – 23S IGS regions appeared to be well conserved within each species but significantly variable between related bacterial species, ideal to design species-specific PCR primers targeting variable regions within the 16S – 23S IGS region. The 16S – 23S IGS region was found to be exclusive enough to make several primer sets for *F. psychrophilum* and two for both *T. maritimum* and *T. columnare*. To test every primer pair combination possible, each primer was tested against all possible primers from other primer pairs.

It was not possible to construct primers for *T. soleae* and *T. branchiophilum* targeting the 16S – 23S IGS region (Table 6). The target sequences of all primers are shown in Appendix II.

16S – 23S IGS primer pairs were used for amplification at described °Tm by Primer3 software (Table 6) (PCR protocol 4). DNA from two reference strains was amplified; *F. psychrophilum* and *T. maritimum,* as well as DNA from Icelandic *F. psychrophilum* isolates and other sample isolates used for negative control. When target sequences were amplified with 16S – 23S IGS primers some species-non-specific amplification was recorded, see dark grey boxes (Table 6).

Table 6. List of primer pairs constructed in this study targeting the 16S – 23S IGS region and PCR amplification results. Tm = Optimal melting temperature of primers. + = amplification of product, - = no amplification of product. MAR = *T. maritimum*, SOL = *T. soleae*, MOR = *Moritella viscosa*.

						Icelandic *F. Psychrophilum* isolates					Reference strains		Negative controls						
Target bacteria	**Primer pair**	**Forward primer**	**Reverse Primer**	**Product size (bp)**	**°Tm (°C)**	**60**	**62**	**19**	**169**	**140**	**SOL**	**MAR**	**379**	**349**	**214**	**172**	**94**	**16**	**MOR**
F. psychrophilum	6	Psy-3F	Psy-3R	551	60	+	-	+	+	+	+	-	-	+	+	+	+	+	+
	7	Psy-4F	Psy-4R	562	60	-	-	-	+	+	+	-	-	+	+	+	-	+	-
	8	Psy-5F	Psy-5R	396	57	+	-	-	+	+	-	+	-	-	-	-	-	-	-
	9	Psy-3F	PSY-4R	559	60	+	-	-	+	+	+	-	-	+	+	+	-	+	-
	10	Psy-4F	Psy-3R	554	60	-	-	+	+	+	+	-	-	+	+	+	-	+	-
	11	Psy-3F	Psy-5R	512	57	+	-	+	+	+	+	-	-	+	+	+	+	+	+
	12	Psy-5F	Psy-3R	434	57	+	-	-	+	+	-	+	-	-	-	-	+	-	-
	13	Psy-4F	Psy-5R	515	57	+	-	+	+	+	+	-	-	+	+	-	+	+	+
	14	Psy-5F	PSY-4R	442	57	-	-	-	+	+	-	-	-	-	-	-	-	-	-
F. columnare	15	Col-1F	Col-1R	945	59	-	+	-	+	-	-	+	-	-	+	-	-	-	+
	16	Col-2F	Col-1R	846	60	+	+	+	+	+	+	+	+	+	+	+	+	-	-
T. maritimum	17	Mar-1F	Mar-1R	162	57	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	18	Mar-1F	Mar-2R	162	59	-	-	+	-	-	-	-	-	-	+	-	-	-	-

After testing IGS primers with the recommended annealing temperature, three primer pairs were chosen for continued testing, due to promising results; primer pairs 8, 9 and 17 (Table 6). Sequences from three reference strains were amplified; *F. psychrophilum, T. maritimum* and *T. soleae*, as well as strains of *F. branchiophilum* and *F. columnare* (PCR protocol 4).

When DNA sequences were amplified with primer pair 17, Mar-1F – Mar-1R, specific amplification for *T. maritimum* was recorded at 55°C (initial annealing temperature) (Figure 5, lane 13). Figure 5 is a compilation of two gel pictures.

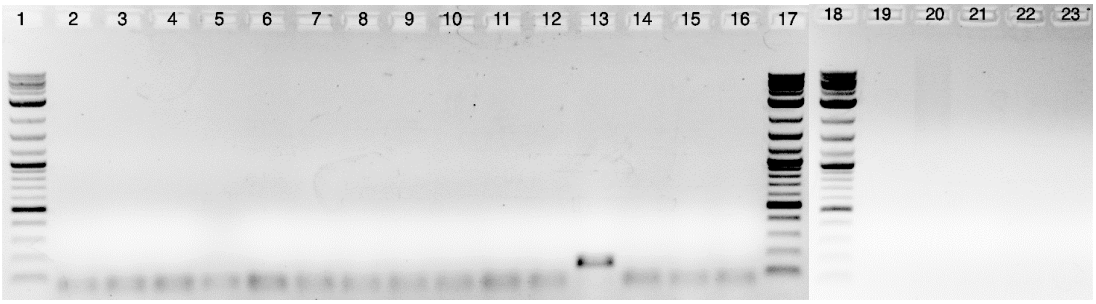


Figure 5. Results showing the amplification with primer pair 17 (Mar-1F – Mar-1R) (*T. maritimum)*.
Lanes: 1, 17 and 18 2-log ladder. Lanes 2, 7, 12 and 19 = *F. psychrophilum,* lanes 3, 8, 13 and 20 = *T. maritimum,* lanes 4, 9, 14 and 21 = *T. soleae,* lanes 5, 10, 15 and 22 = *F. branchiophilum,* lanes 6, 11, 16 and 23 = *F. columnare.* Lanes 2-6 = amplification at 51°C, 7-11 = amplification at 53°C, 12-16 amplification at 55°C, 19-23 = amplification at 57°C. °C = initial annealing temperature.

When sequences were amplified with primer pair 9, Psy-3F – Psy-4R, some species-non-specific amplification was recorded, see lanes with an asterisk (Figure 6). Figure 6 is a compilation of two gel pictures.

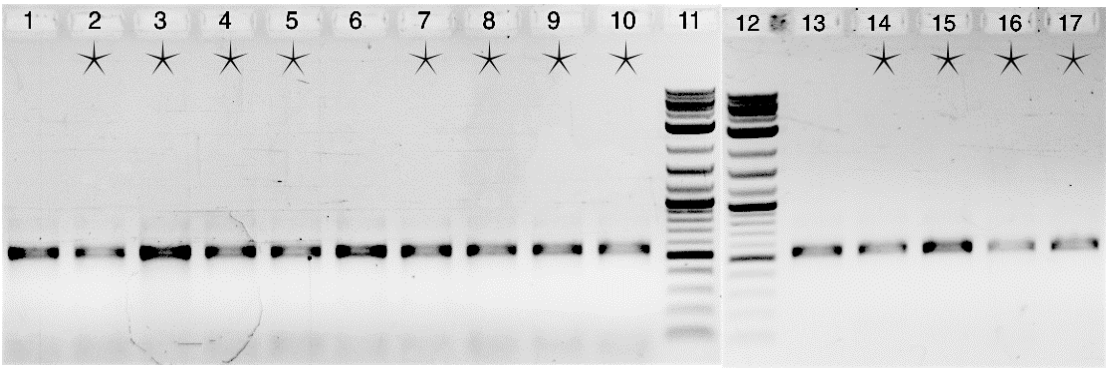


Figure 6. Results showing the amplification with primer pair 9 (Psy-3F – Psy-4R) (*F. psychrophilum*).
Lanes: 11 and 12 2-log ladder. Lanes 1, 6 and 13 = *F. psychrophilum.* Lanes 2, 7 and 14 = *T. maritimum*, lanes 3, 8 and 15 = *T. soleae,* lanes 4, 9 and 16 = *F. branchiophilum,* lanes 5, 10 and 17 = *F. columnare.* Lanes 1-5 = amplification at 59°C, 6-10 = amplification at 61°C, 13-17 = amplification at 63°C. °C = initial annealing temperature. Lanes marked with an asterisk indicate species-non-specific amplification.

When sequences were amplified with primer pair 8, Psy-5F – Psy-5R, specific amplification for *F. psychrophilum* was recorded at 59°C (initial annealing temperature) (Figure 7, lane 6). Figure 7 is a compilation of two gel pictures.

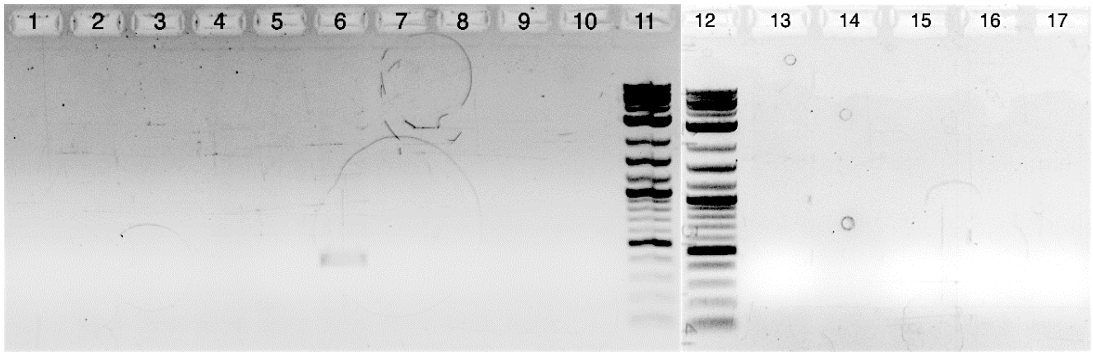


Figure 7. Results showing the amplification with primer pair 8 (<u>Psy-5F – Psy-5R</u>) (*F. psychrophilum*).
Lanes: 11 and 12 2-log ladder. Lanes 1, 6 and 13 = *F. psychrophilum,* lanes 2, 7 and 14 = *T. maritimum,* lanes 3, 8 and 15 = *T. soleae,* lanes 4, 9, and 16 = *F. branchiophilum,* lanes 5, 10 and 17 = *F. columnare*. Lanes 1-5 = amplification at 57°C, 6-10 = amplification at 59°C, 13-17 = amplification at 61°C. °C = initial annealing temperature.

Due to promising species-specific amplification, primer pair 8, Psy-5F – Psy-5R, was tested further. Sequences from three reference strains were amplified; *F. psychrophilum, T. maritimum* and *T. soleae* as well as *F. psychrophilum* sample isolates, selected based on their phylogenetic relationship to the *F. psychrophilum* reference strain and Icelandic *F. psychrophilum* isolates (data not shown).

When samples were amplified with primer pair 8, some *F. psychrophilum* isolates did not amplify, see lanes with an arrow (Figure 8).

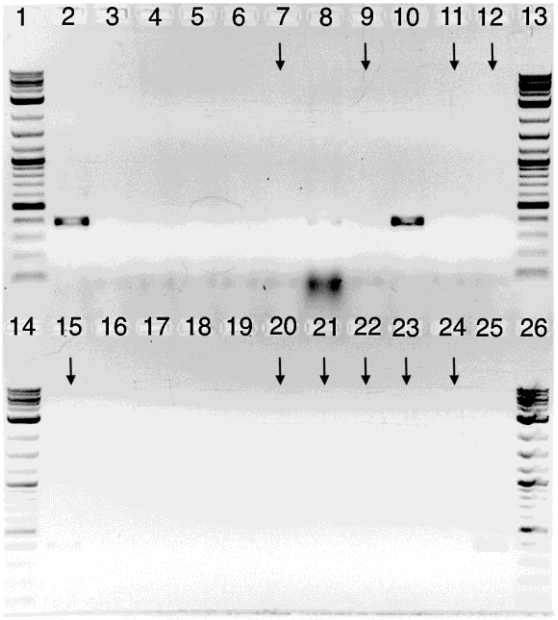


Figure 8. Results showing the amplification with primer pair 8 (Psy-5F – Psy-5R) (*F.psychrophilum*).
Lanes: 1, 13, 14 and 26 2-log ladder. Lanes 2 and 15 = *F. psychrophilum,* lanes 2 and 16 = *T. maritimum,* lanes 3 and 17 = *T. soleae,* lanes 4 and 18 = *F. branchiophilum,* lanes 5 and 19 = *F. columnare,* lanes 7-12 and 20-25 = *F. psychrophilum* isolates in this study. Lanes 2-12 = amplification at 59°C, 15-25 = amplification at 61°C. °C = initial annealing temperature. Lanes marked with an arrow indicate missing species-specific amplification.

4.3 Identification of tail and fin rot bacteria using *F. psychrophilum gyrB* nested primers and *T. soleae* 16S – 23S IGS region primers

Due to inconsistent amplification of *F. psychrophilum* using primer pair 8 (Psy-5F – Psy-5R) targeting the 16S – 23S IGS region and no primer pair being specific enough to be used for the detection of *T. soleae*, previously published primers targeting other genes were tested.

Species-specific primers PSY-G1F – PSY-G1R for *F. psychrophilum* targeting the *gyr B* gene were giving a 1017 bp PCR product (91) (PCR protocol 3). Three reference strains, *F. psychrophilum, T. maritimum* and *T. soleae*, were amplified with universal gyrB primers: GYR1 – GyrR, which give a 1178 bp PCR product. Next, various dilutions of gyrB universal PCR product were amplified using the PSY-G1F and PSY-G1R nested primers. The nested PCR results gave species-non-specific amplification where both *T. maritimum* and *T. soleae* were amplified. No further testing was carried out with these primers.

Species-specific primers, G47F – G47R, for *T. soleae,* target the 16S – 23S IGS region and give a 1555 bp product (112) (PCR protocol 2). The 16S – 23S IGS region of three reference strains was amplified with G47F – G47R; *F. psychrophilum, T. maritimum* and *T. soleae,* as well as strains of *F. branchiophilum* and *F. columnare.* The outcome was promising as the amplification of *F. psychrophilum* and *T.* maritimum became more faint with increasing annealing temperature, whereas the *T.* soleae band remained well visible.

In order to obtain better specificity of primers G47F – G47R, adjustments were made to the $MgCl_2$ levels in the reaction buffer (PCR protocol 2). Three reference strains were amplified; *F. psychrophilum, T. maritimum* and *T. soleae.* Still a species-non-specific amplification was recorded as *T. maritimum* amplified as well as *T. soleae*. Next the PCR conditions were optimized further, both adjustment of annealing temperature and DNA concentration (Table 7). Only the *T. maritimum* and *T. soleae* reference strains were amplified, as *F. psychrophilum* did not amplify with the $MgCl_2$ concentration adjusted. Now a specific amplification of *T. soleae* was recorded at 63°C (initial annealing temperature) (Figure 9). Figure 9 is a compilation of 3 gel pictures.

Table 7. Names of samples with various temperature, DNA concentration and $MgCl_2$ levels. The names of samples correspond to lane numbers in Figure 9. Temperatures used in the table are annealing temperatures.

		Lanes							
		57°C		59°C		61°C		63°C	
$MgCl_2$ (mM)	DNA concentration (~ng)	*T. maritimum*	*T. solea*	*T. maritimum*	*T. solea*	*T. maritimum*	*T. solea*	*T. maritimum*	*T. solea*
0	100	2	4	14	16	28	30	42	44
	50	3	5	15	17	29	31	43	45
1.25	100	6	8	18	22	32	34	46	48
	50	7	9	19	23	33	35	47	49
2.50	100	10	12	24	26	36	38	50	52
	50	11	13	25	27	37	39	51	53

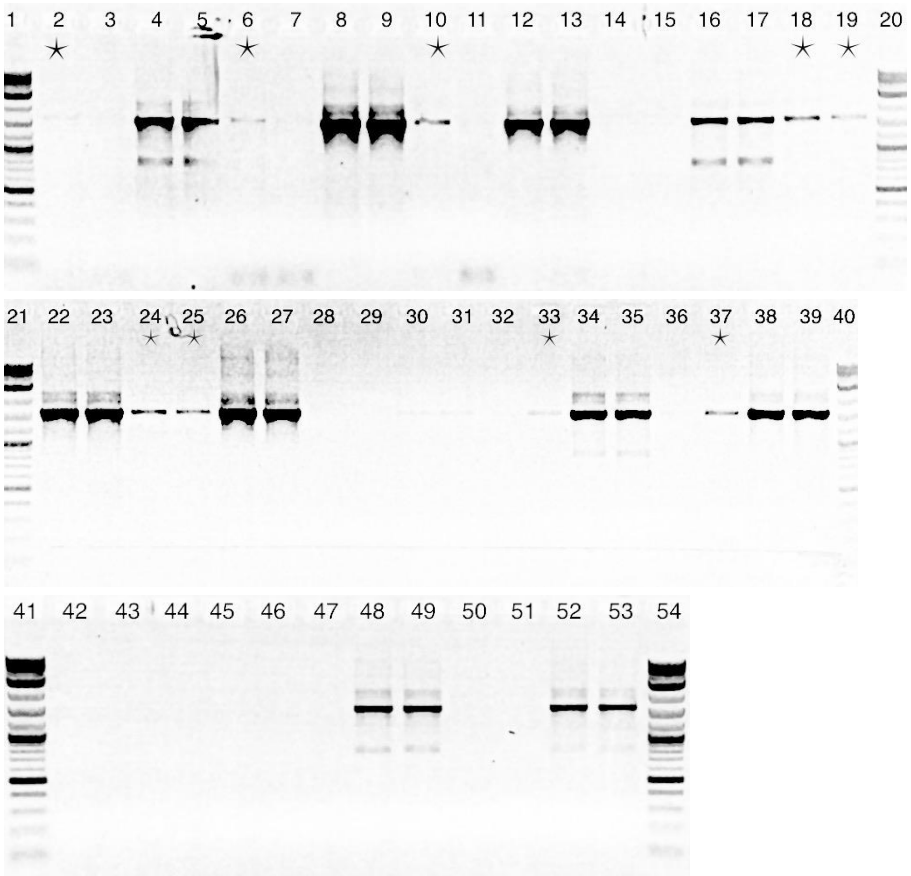


Figure 9. PCR amplification with PCR primers G47F – G47R with various levels of $MgCl_2$, DNA concentration and temperature. Lanes: 1, 20, 21, 40, 41, 54 2-log ladder. Lanes 2-53 *T. maritimum* and *T.* soleae PCR product with different $MgCl_2$ and DNA concentration and variable annealing temperature (Table 7.). Lanes marked with an asterisk indicate species-non-specific amplification.

4.4 Phylogenetic study

To examine the genetic relationship between Icelandic tail and fin rot isolates and *F. psychrophilum, T. maritimum* and *T. soleae* reference strains, 1147 bp sequences of the 16S rRNA gene were aligned, from 214 isolates and 3 reference strains in total. All gaps and inconclusive bases were eliminated from the analysis using MUSCLE alignment.

The phylogenetic tree shows single isolates as well as groups of identical sequences (Figure 10). The primary focus was to compare Icelandic *Flavobacterium* strains to the reference strains, as the isolates may be the causative agents of tail and fin rot in aquaculture. Figure 10A presents the relationship within *Flavobacterium* strains using the neighbor-joining method. Figure 10B shows full bootstrap support values. It is evident both in Figures 11A and 11B that there is considerable variability within *Flavobacterium* strains in Iceland.

However, there is great similarity between the *F. psychrophilum* reference strain and groups containing a large part of the Icelandic tail and fin rot bacterial isolates used in the phylogenetic study (~32%): H21 group (29 isolates), H22 group (20 isolates) and H16 group (19 isolates), most of which originate in Arctic charr.

The horizontal line at the bottom of figure 10 shows the scale bar and it is used to give rough measurement of genetic distances. If the aim is to find the genetic distance between two species, the horizontal distances between the species of interest are added and the total number is compared to the scale bar (101). The scale bar indicates that the relationship between the *F. psychrophilum* reference strain and the other *F. psychrophilum* isolates falls within 0.035 limits, meaning at least 96,5% similarity of sequences.

Furthermore, different types of samples tend to cluster together (Figure 11). Most water samples are situated at the top of the tree (red circle) whereas roe (yellow circle) and fish (green circle) samples are at the bottom of the tree. Some strains are represented as single groups with 100% bootstrap assembly while others are distinct strains. Other different types of phylogenetic trees made from the same data are included in Appendix III. For detailed information on samples and tree construction settings, see Appendix IV.

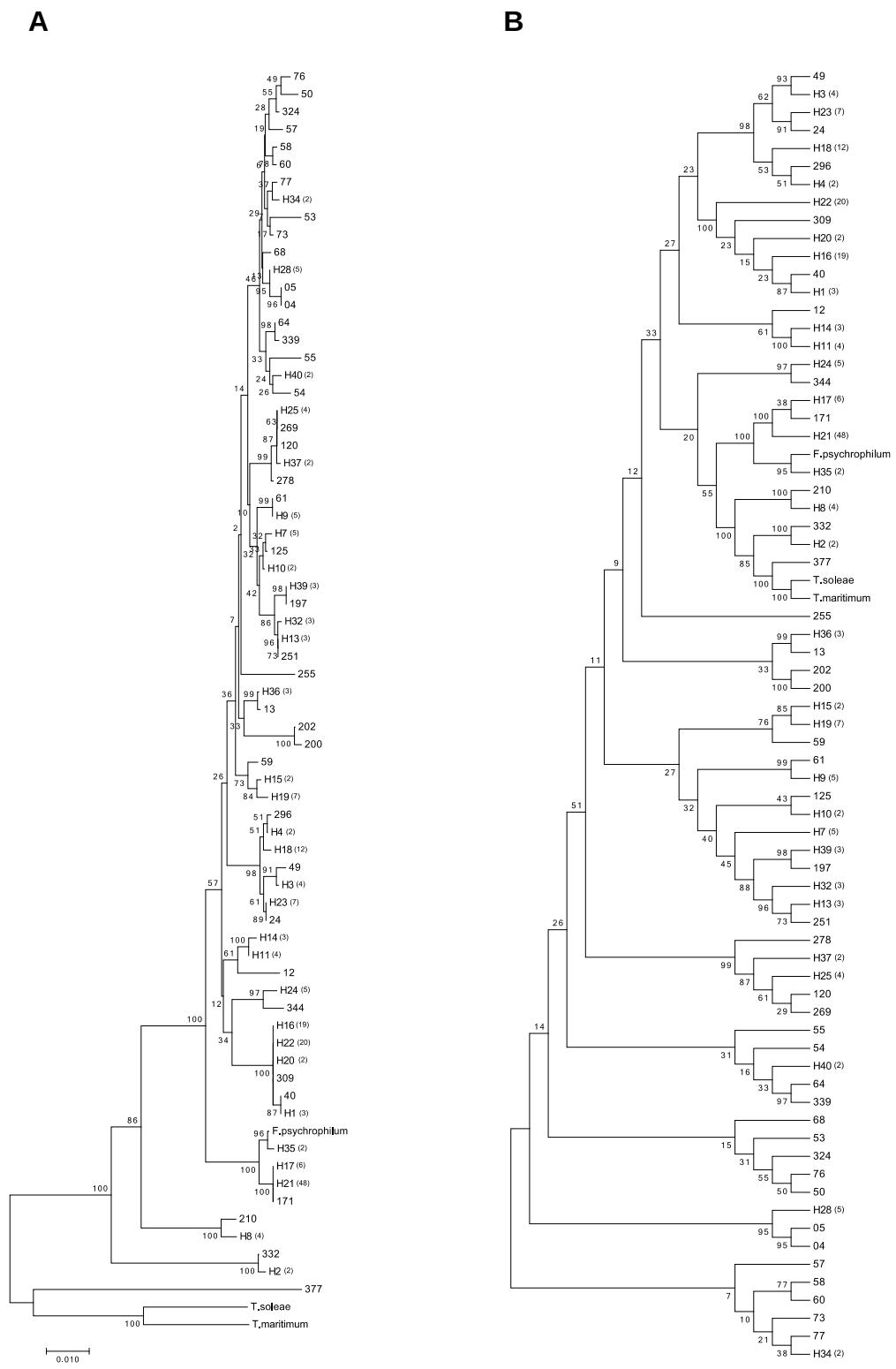


Figure 10. Relationship within Icelandic *Flavobacterium* strains using the neighbor-joining method. Relationship based on a 1147 bp 16S rRNA sequence analysis from 214 isolates and 3 reference strains using neighbor-joining method with 500 bootstrap replications. Single strains are represented with a number and a group of strains with identical 16S rRNA sequences is represented with an H-name. The scale bar is 0.01 and the sum of branch length is 0.472. The numbers by the branches show the confidence limits estimated by bootstrap analysis. A: Optimal tree with bootstrap values B: Bootstrap consensus tree with no cutoff.

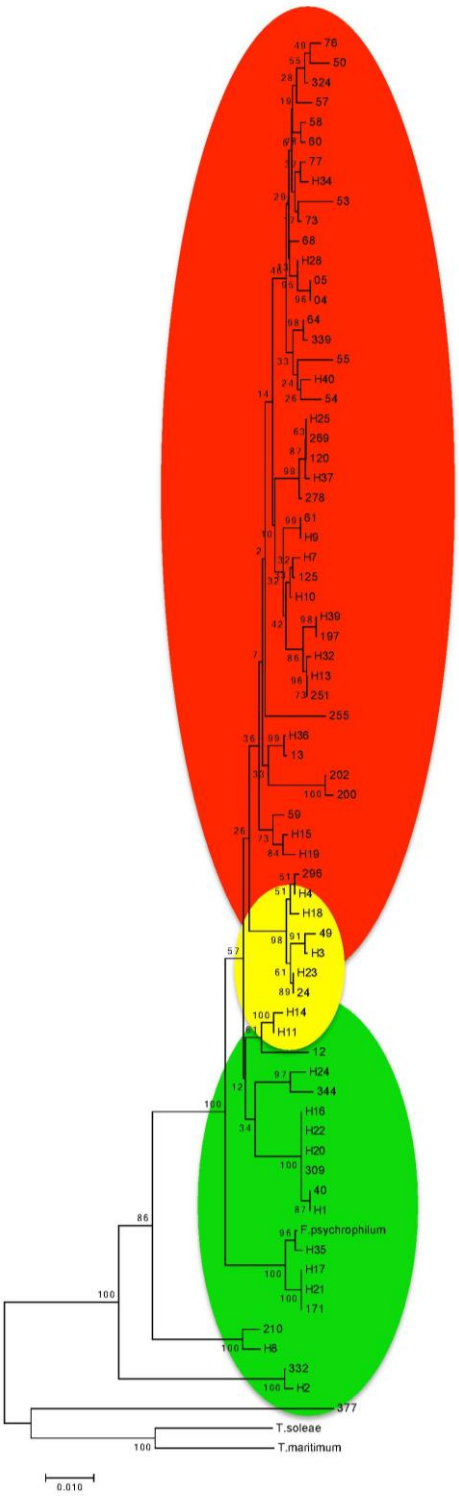


Figure 11. Schematic figure of sample distribution within Icelandic *Flavobacterium* isolates. Most water samples are within the red circle, roe samples are within the yellow circle and within the green circle are isolates sampled from diseased fish.

4.5 Measuring bacterial growth

The temperature and salinity tolerance for *F. psychrophilum, T. maritimum* and *T. soleae* reference strains and Icelandic bacterial isolates was measured by examining bacterial growth in FMM medium when the temperature and salinity were varied. In order to obtain trustworthy results, the temperature and salinity tolerance tests were replicated (Figures 13 and 14 represent Test 1 and Test 2 of reference strains and figures 15 and 16 represent Test 1 and Test 2 of the Icelandic isolates).

Pure cultures of bacteria were cultured at three different salinity levels; 0 (●), 16 (■) and 32 (▲) ppm for five days with three replicates for each treatment. Bacterial density was measured as OD values at 600 nm.

FMM medium containing freshwater (0 ppm) was best suited for *F. psychrophilum* growth. *T. maritimum* grew almost equally well in medium containing 16 (brackish water) and 32 ppm (seawater), with 32 ppm being slightly more suitable. The growth of *T. soleae* was the most prominent of all three bacterial species, of which, FMM containing 32 ppm (seawater) was the most effective medium (Figure 12). Statistical significance ($p<0.0001$) was obtained using two-factor ANOVA for both time factor and treatment factor resulting in significant growth difference between day 0 and day 4 for bacterial strains treated.

For a continuing study, 13 Icelandic bacterial isolates containing 7 Artic charr isolates, 2 Atlantic salmon isolates and one isolate each from lumpfish, rainbow trout, haddock and redfish were measured against 3 different salinity levels as described above (Figure 14).

Salinity tolerance test 1 made on Icelandic bacteria isolates revealed more variable results than the *Flavobacterium* reference strains. Culture in liquid media was not suitable for 3 of the Artic charr isolates: #1, #4 and #5 and were therefore not used in a replicated study.

Medium containing freshwater (0 ppm) was best suitable for most Artic charr isolates but medium containing brackish water (16 ppm) was more suitable for Artic charr isolates #3 and #7. Only Artic charr isolate #6 was able to grow on medium containing seawater (32 ppm). Culture of Artic charr isolates #3 and #7 was also possible on 32 ppm medium but only in the replicated study, salinity tolerance test 2. The growth of Atlantic salmon isolates was most prominent at 0 ppm but culture was also possible in medium containing brackish water and seawater. Culture in medium containing seawater was more suitable for lumpfish, rainbow trout, haddock and redfish isolates than brackish water and culture in medium containing freshwater (0 ppm) was very limited.

In salinity tolerance test 1 a two-factor ANOVA test revealed a statistical significance for both time factor and treatment factor resulting in significant growth difference between day 0 and day 4 of all bacteria tested except isolates Artic charr #1, #4 and #5 were no growth was detected. The statistical significance value for both time factor and treatment factor was $p<0.0001$ for most isolates but the significance value for the treatment factor was $p=0.0005$ for isolate Redfish #1.

The salinity tolerance test 2 revealed same significant results as test 1 ($P<0.0001$) using a two-factor ANOVA test for both time factor and treatment factor except isolates Artic charr #3, Artic charr #7 and Redfish #1 where the treatment factor was $p=0.0032$, 0.0011 and 0.0017 respectively.

Following the salinity experiment, temperature tolerance was tested for *F. psychrophilum, T. maritimum* and *T. soleae* reference strains (Figure 13) as well as Icelandic *Flavobacterium* isolates (Figure 15). Reference strains and Icelandic isolates were cultured in medium most suitable for their growth, outcome from salinity tolerance test. *F. psychrophilum*, Artic charr isoates 1, 2, 4, 5 and 6 and Atlantic salmon isolates were cultured in FMM using ddH_2O (0 ppm), Artic charr isolates 3 and 7 were cultured using FMM with brackish water (16 ppm) and *T. maritimum*, *T. soleae* and isolates of Lumpfish, rainbow trout, haddock and redfish were cultured using FMM with seawater (32 ppm). Culture was carried out at three different temperatures; 5°C (●), 10°C (■) and 15°C (▲). Bacterial growth was measured as OD values at 600 nm.

The optimal temperature for all *Flavobacterium* reference strains was 15°C, with *T. soleae* growing the fastest. All three strains grew the least at 5°C, with no detectable growth of *T. maritimum* (Figure 13). Statistical significance ($p<0.0001$) was obtained using two-factor ANOVA test for both time factor and treatment factor resulting in significant growth difference between day 0 and day 4 and between salinity treatments for bacterial strains treated.

The optimal temperature for all Icelandic bacterial isolates was 15°C with isolates rainbow trout #1 and haddock #1 growing the fastest. Growth was barely detectable at 5°C for Artic charr isolate #2, but no growth was detected for isolates Artic charr #1, #4 and #5 (Figure 15).

In temperature tolerance test 1, a two-factor ANOVA test revealed statistical significance for both time factor and treatment factor resulting in significant growth difference between day 0 and day 4 of all bacteria tested except isolates Artic charr #4 and Artic charr #5, were no growth was detected.

The statistical significance value for both time factor and treatment factor was $p<0.0001$ for most isolates but the significance value for the treatment factor was $p=0.0203$, 0.0003 and 0.0003 for isolates Artic charr #7, Haddock #1 and Redfish #1 respectively.

Isolates Artic charr #1, #4 and #5 were not cultured for salinity tolerance test 2 due to low cell count in the previous test.

The salinity tolerance test 2 revealed same significant results as test 1 ($P<0.0001$) using a two-factor ANOVA for both time factor and treatment factor except isolate haddock #1 where the treatment factor was $p=0.0024$.

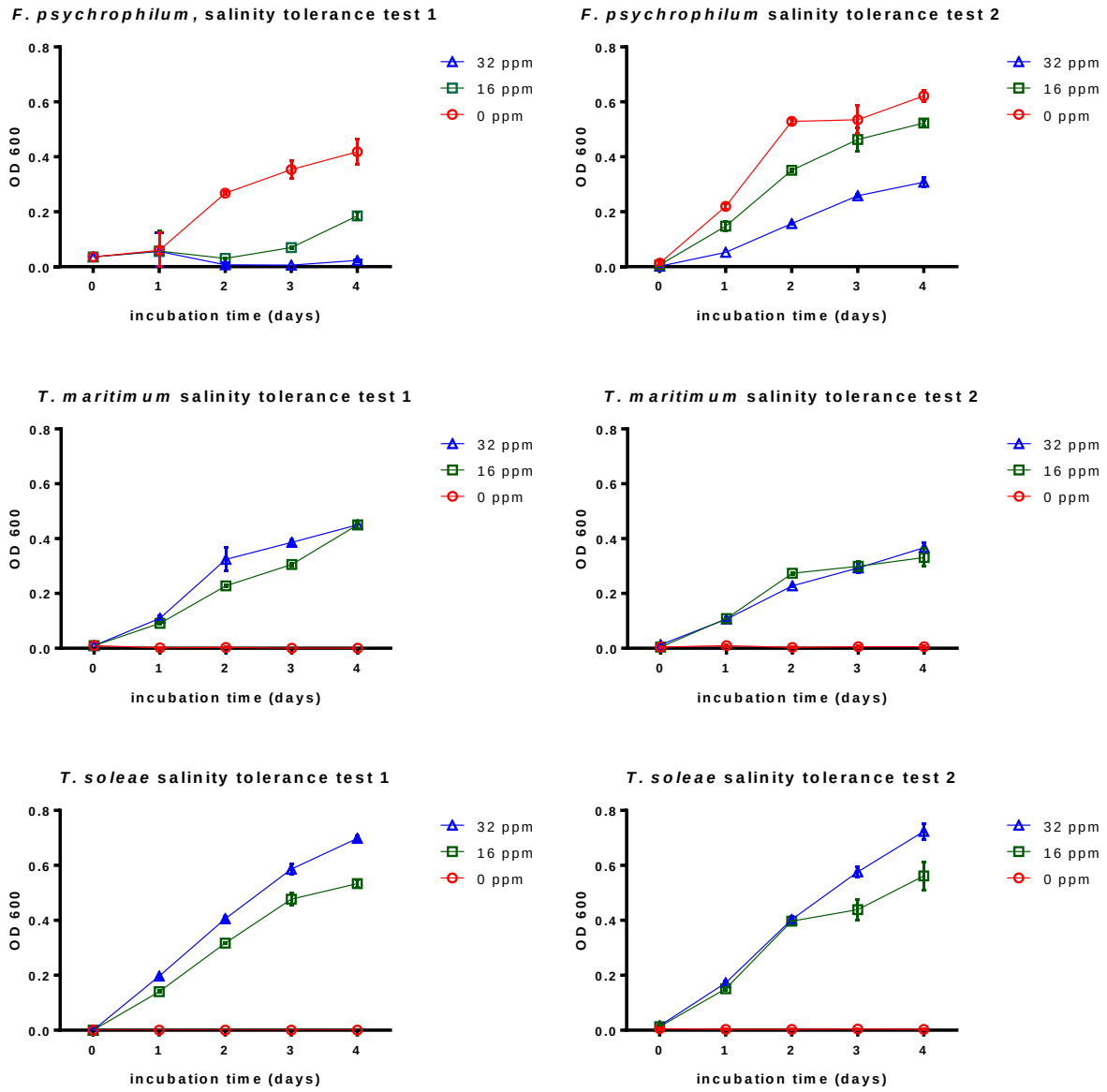


Figure 12. Bacterial growth of *F. psychrophilum, T. maritimum* and *T. soleae* at different salinity levels. Y-axis: OD at 600 nm X-axis: incubation time in days. Lines represent mean +/- standard error of mean.

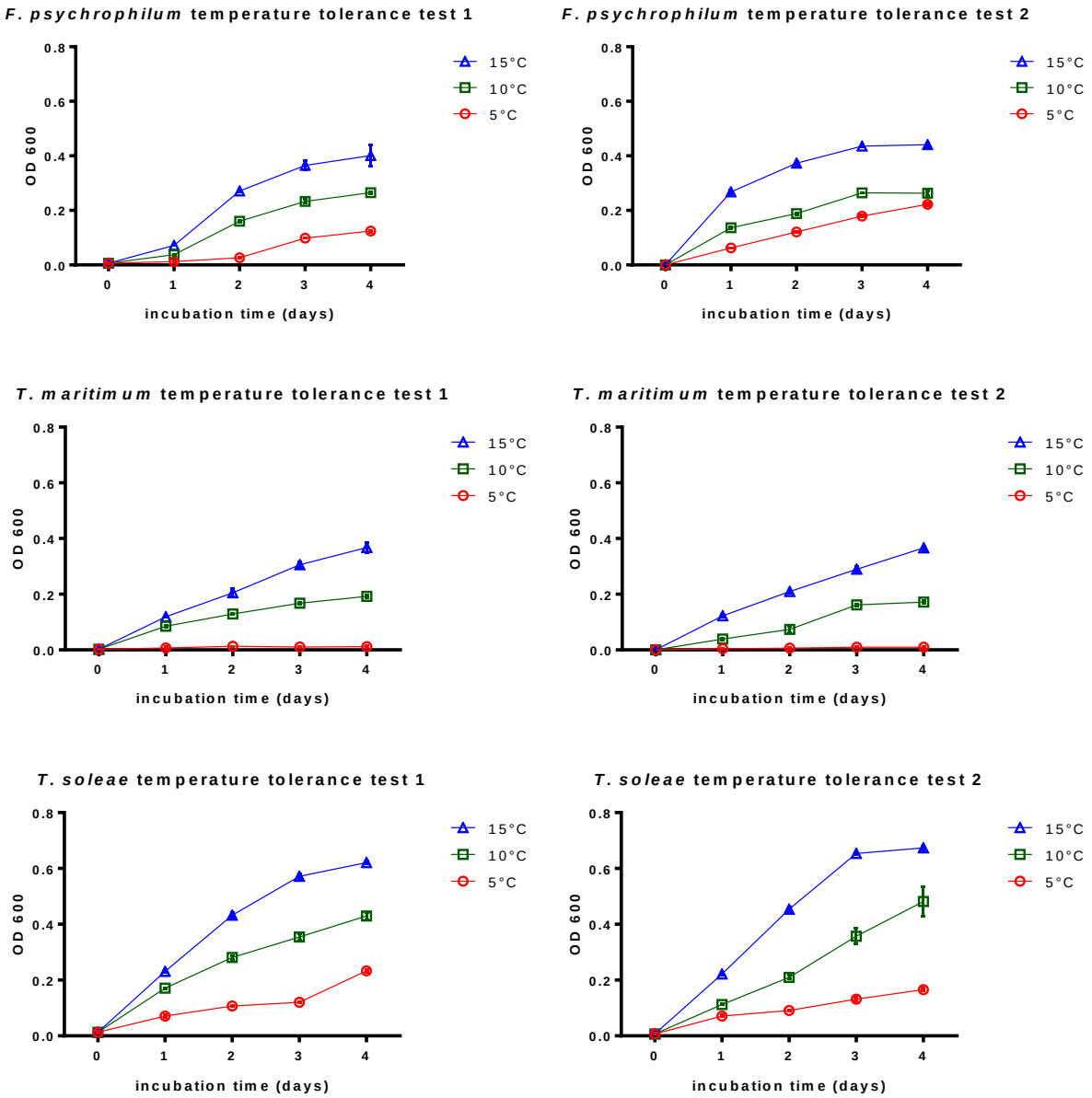
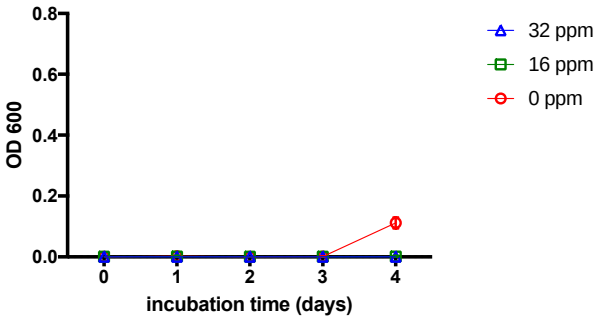
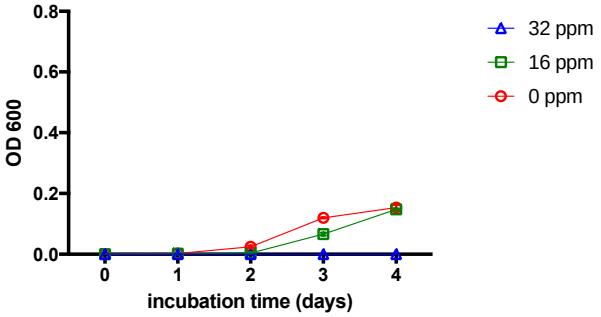


Figure 13. Bacterial growth of *F. psychrophilum*, *T. maritimum* and *T. soleae* at different temperatures. Y-axis: OD at 600 nm X-axis: incubation time in days. Lines represent mean +/- standard error of mean.

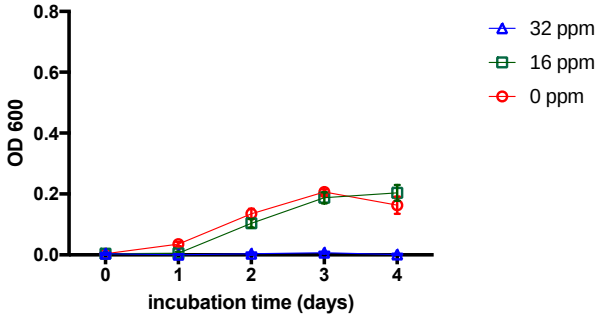
Artic charr #1 salinity tolerance test 1



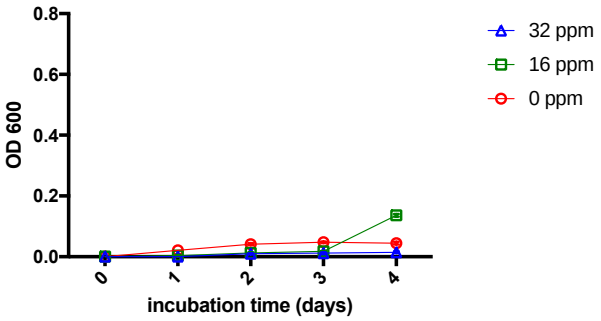
Artic charr #2 salinity tolerance test 1



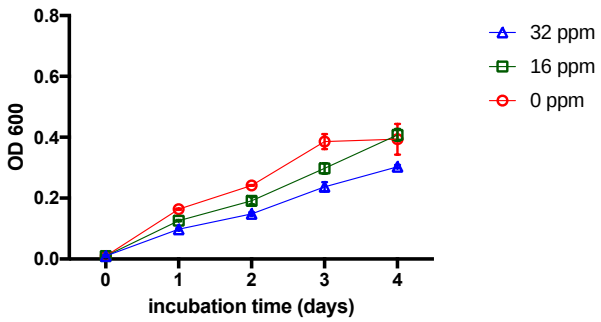
Artic charr #2 salinity tolerance test 2



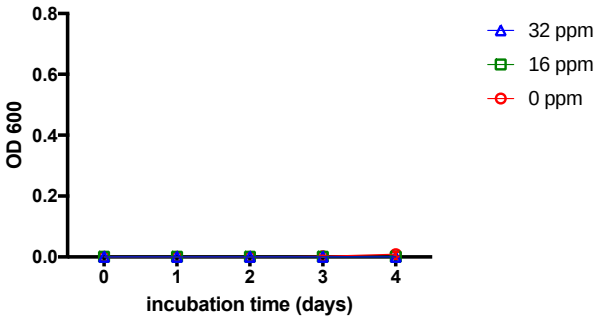
Artic charr #3 salinity tolerance test 1



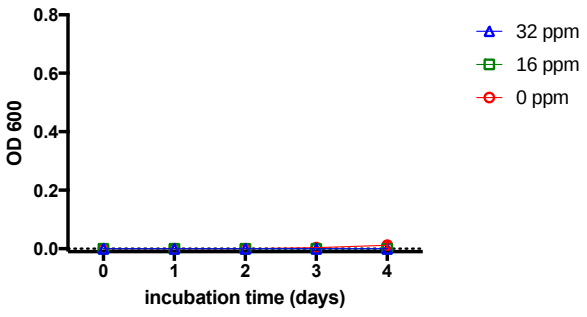
Artic charr #3 salinity tolerance test 2

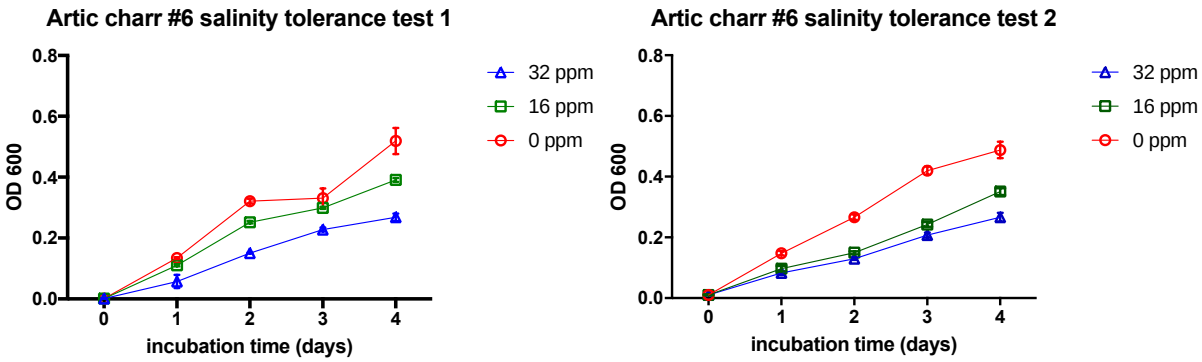


Artic charr #4 salinity tolerance test 1

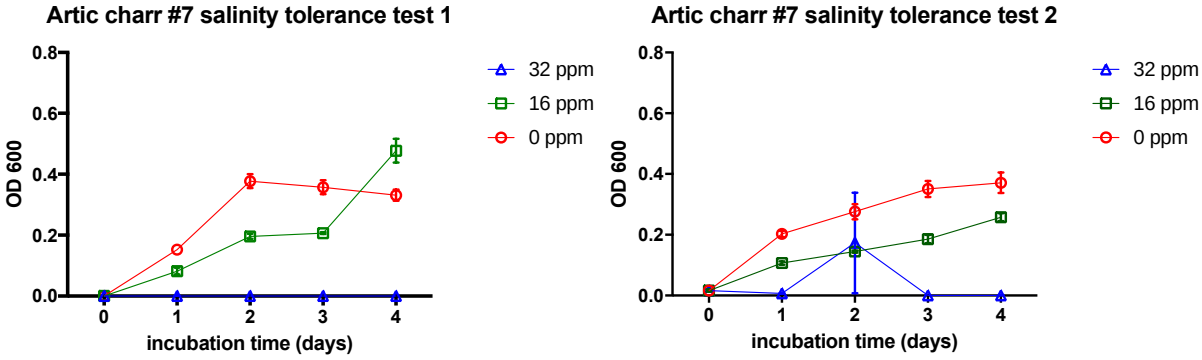


Artic charr #5 salinity tolerance test 1

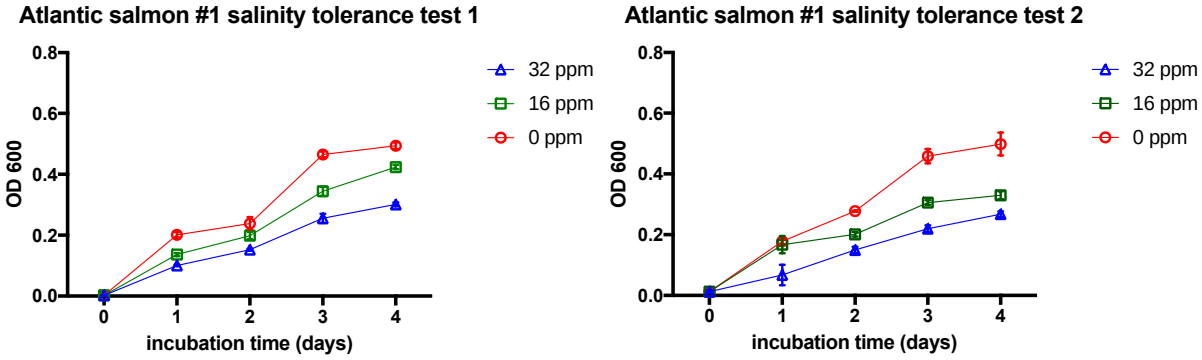



Artic charr #6 salinity tolerance test 1
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm

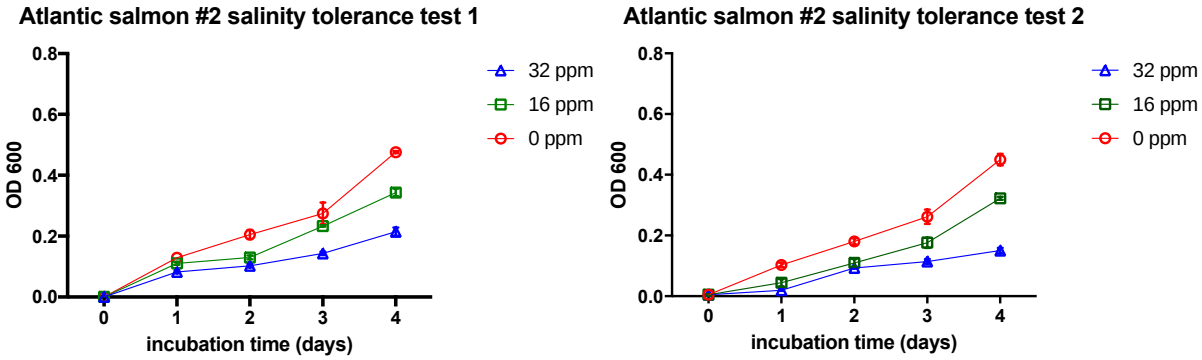
Artic charr #6 salinity tolerance test 2
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm


Artic charr #7 salinity tolerance test 1
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm

Artic charr #7 salinity tolerance test 2
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm


Atlantic salmon #1 salinity tolerance test 1
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm

Atlantic salmon #1 salinity tolerance test 2
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm


Atlantic salmon #2 salinity tolerance test 1
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm

Atlantic salmon #2 salinity tolerance test 2
OD 600
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
incubation time (days)
32 ppm
16 ppm
0 ppm

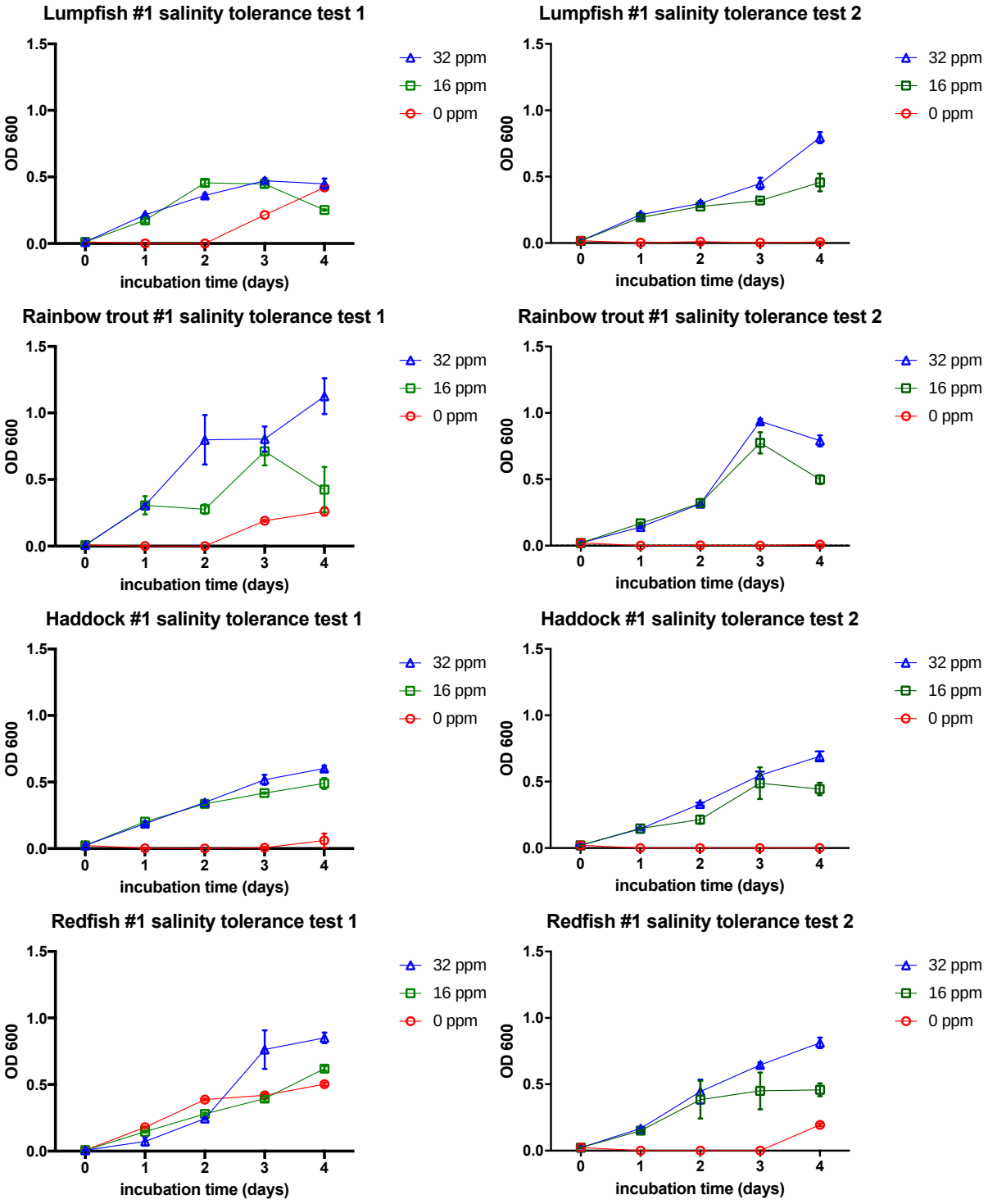
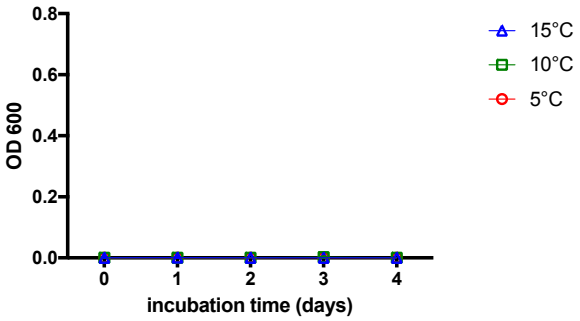
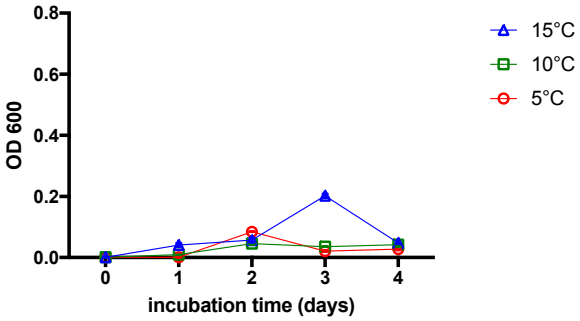


Figure 14. Bacterial growth of Icelandic *Flavobacterium* isolates at different salinity levels. Y-axis: OD at 600 nm X-axis: incubation time in days. Lines represent mean +/- standard error of mean.

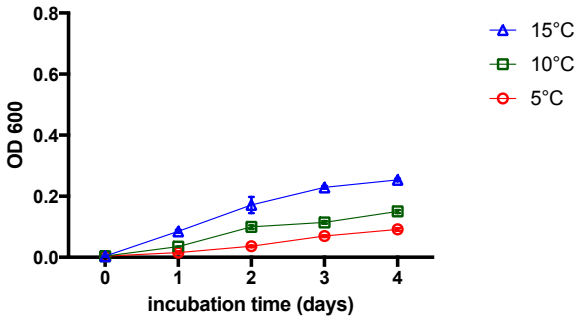
Artic charr #1 temperature tolerance test 1



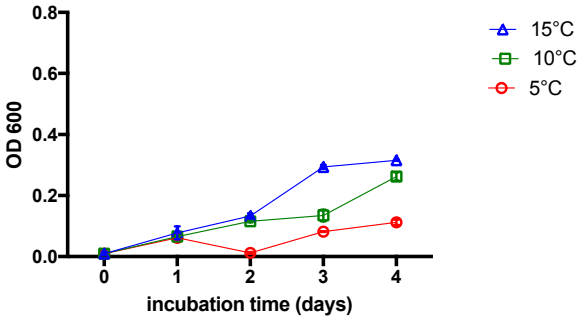
Artic charr #2 temperature tolerance test 1



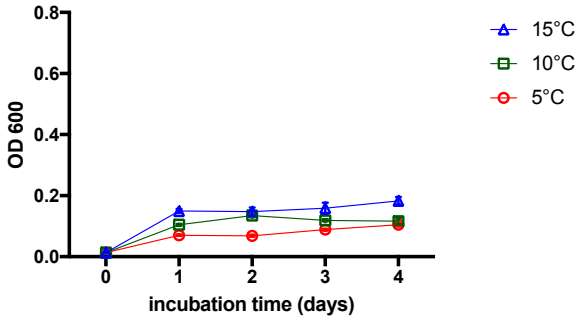
Artic charr #2 temperature tolerance test 2



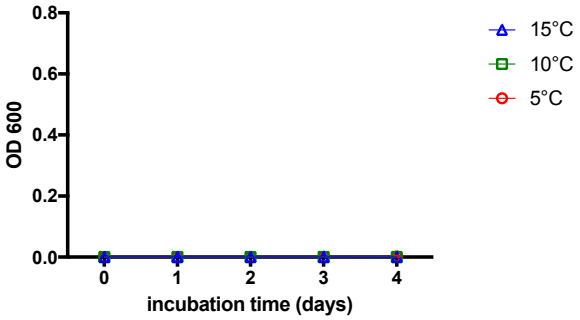
Artic charr #3 temperature tolerance test 1



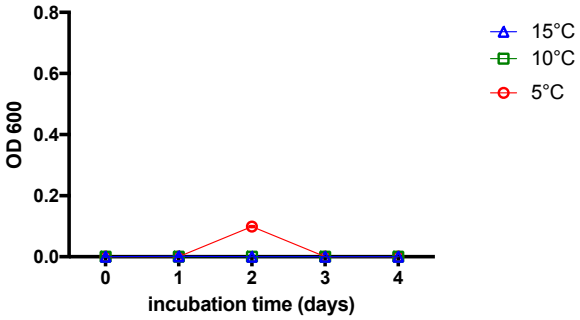
Artic charr #3 temperature tolerance test 2



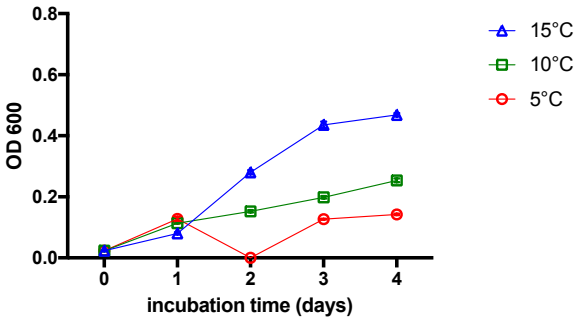
Artic charr #4 temperature tolerance test 1



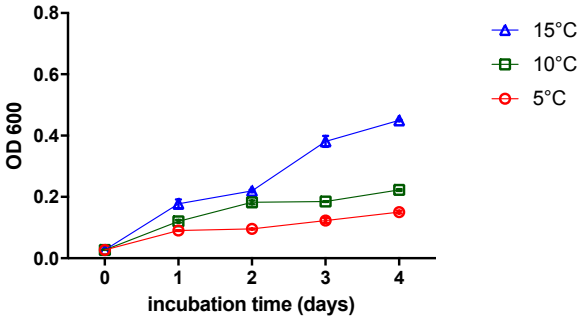
Artic charr #5 temperature tolerance test 1



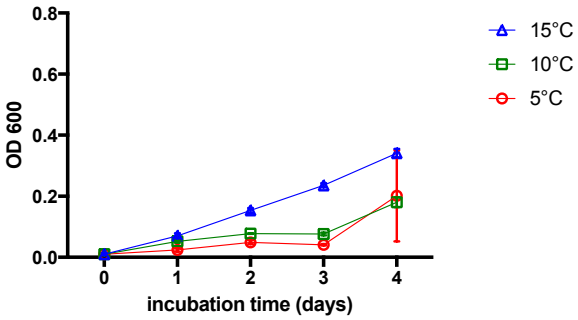
Artic charr #6 temperature tolerance test 1



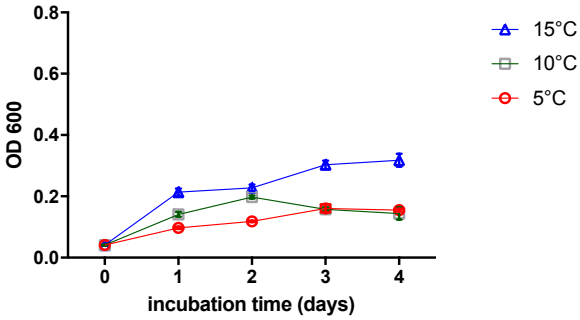
Artic charr #6 temperature tolerance test 2



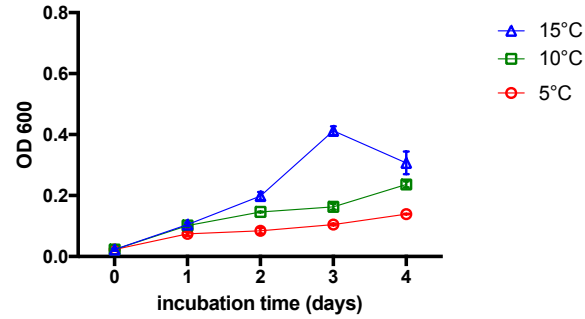
Artic charr #7 temperature tolerance test 1



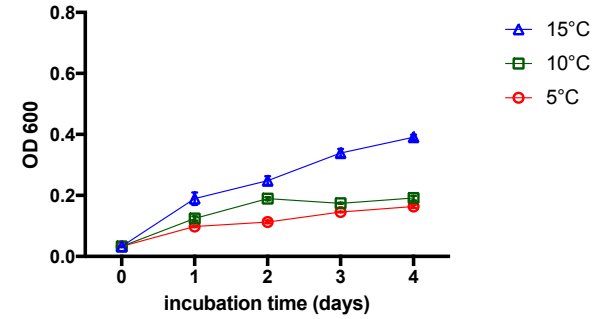
Artic charr #7 temperature tolerance test 2



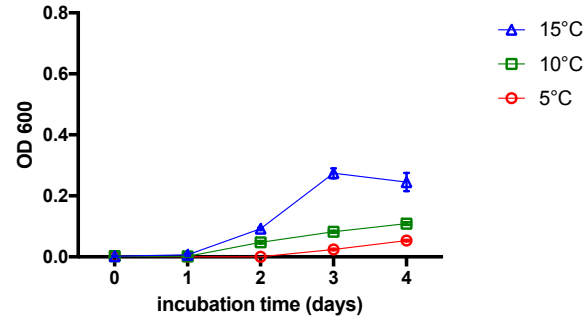
Atlantic salmon #1 temperature tolerance test 1



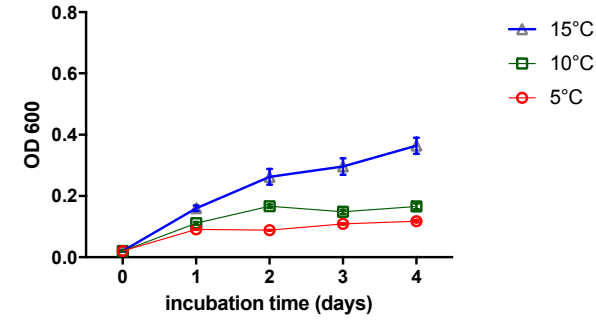
Atlantic salmon #1 temperature tolerance test 2



Atlantic salmon #2 temperature tolerance test 1



Atlantic salmon #2 temperature tolerance test 2



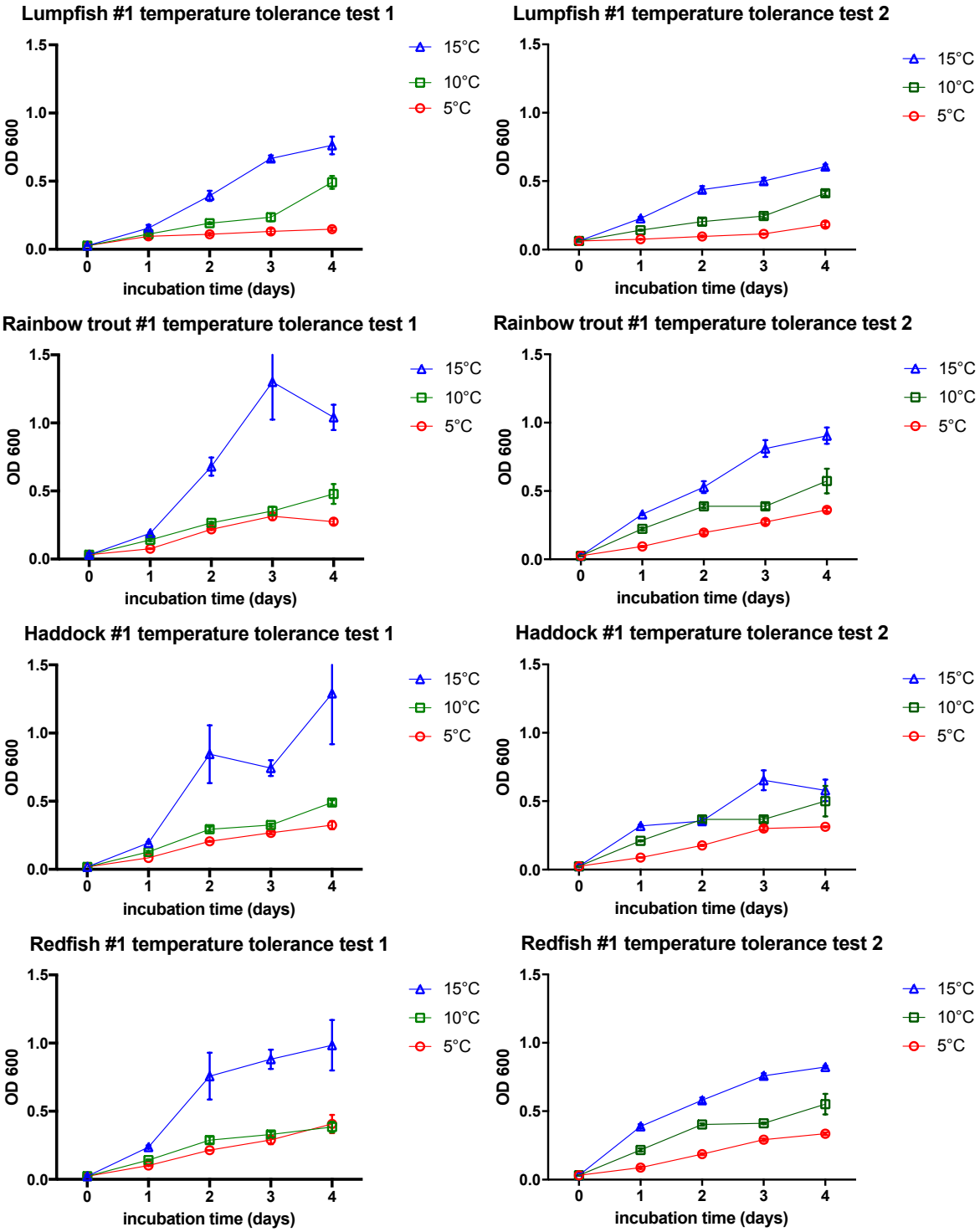


Figure 15. Bacterial growth of Icelandic *Flavobacterium* isolates at different tempatures. Y-axis: OD at 600 nm X-axis: incubation time in days. Lines represent mean +/- standard error of mean.

5 Discussion

Due to known major economical downfalls from tail and fin rot bacteria (2), a fast and reliable identification method is very important to inhibit spreading of disease.

5.1 Bacterial identification using genetic analysis

Determining the type of pathogenic agent is important when it comes to clinical decisions, such as the choice of antibiotics and vaccines. For many years, bacterial identification was based solely on colony morphology, Gram staining and biochemical tests. These methods involve a number of time-consuming steps needed for identification of the organism. PCR, on the other hand, is a highly sensitive technique that produces results rapidly. PCR is an enzymatic assay that can amplify a specific segment of DNA using a small amount of template and specific primers. However, template contamination and non-specific primer binding to sequences with sequence similarity to the target region can lead to amplification of unwanted product (128).

To maximize the efficiency of PCR amplification in this project, a few key factors were tested: 1) Primer3 software was used to design new primers with low risk of primer-dimer formation and non-specific amplification (129). 2) Touchdown PCR protocol was used and the PCR plate was kept on ice until the thermal cycler had reached 80°C. 3) Alteration of $MgCl_2$ concentration in PCR solution.

Primer design is a critical component in determining the sensitivity of PCR (130). Primer length, primer-dimer production, the ratio of AT to GC bases, PCR product length, and placement within the target sequence are some of the most important factors that determine the specificity and efficacy of amplification (131).

Keeping the PCR solution on ice until the PCR cycler reaches at least 80°C during the initial denaturation step, decreases nonspecific amplification by keeping the Taq DNA polymerase inactive until a high temperature is reached. A touchdown PCR allows for increased sensitivity, specificity and yield without redesigning primers and/or rearranging PCR conditions for better results. Touchdown PCR protocol has widely been used when the DNA template is difficult to amplify (117, 118).

Altering of $MgCl_2$ concentration in PCR solution has been used to obtain high species-specific amplification. Both chains of the DNA helix are negatively charged and have, therefore, a natural tendency to repel each other. Positive ions such as Mg^{++} can help fight forces of repulsion and help strengthen the interaction between primers and template DNA. Low $MgCl_2$ concentration can eliminate non-specific priming and high $MgCl_2$ concentration can help stabilize the binding between primers and the target DNA sequence (132).

Partial DNA sequencing and whole genome sequencing of bacteria has rapidly increased due to lowered sequencing cost and rapid turnaround time, and has become a routine tool for microbiology laboratories (133). At present, species are often characterized using both phenotypic and genotypic analysis, but the role of sequencing is steadily gaining weight (133).

The 16S rRNA gene is a well-conserved region within all bacteria due to the critical role that the 16S rRNA plays in cell function. The gene is commonly used for bacterial identification and to estimate

evolutionary distances and relatedness of organisms. The 16S rRNA gene and surrounding regions are large, about 1500 bp long, and are composed of both variable and conserved sequences that generally provide sufficient diversity for differentiating between bacterial strains. However, the usefulness of 16S rRNA gene comparison is limited to species with sufficiently dissimilar sequences (101). Roth *et al.* used the 16S – 23S IGS region to differentiate between *Mycobacterium* species because the bacteria could not be discriminated based on the 16S rRNA gene sequences (134). The 16S – 23S IGS region is however not as widely used as the 16S rRNA gene, and thus offers fewer comparative sequences (101). Other well-conserved genes have also been used to determine species relatedness, such as the 65-kDa heat shock protein (135). MLST, the partial sequencing of various housekeeping genes, has also gained weight in DNA sequence comparison (99).

One major difference between previously described species-specific primers for tail and fin rot bacteria and the primers designed in this study is the choice of negative controls in primer specificity testing. One reason for this is how recently some *Flavobactericeae* species were discovered. *T. soleae*, for example, was first described in 2008 (3, 105, 136). According to our results, the 16S rRNA genes of *T. soleae* and *T. maritmum* are very similar and that is likely to affect the specificity of primers. In other papers, where new primers have been described, authors often leave out closely related bacterial strains when choosing negative controls when the specificity of primers is tested (91, 106, 108, 109, 111). This will of course continue to be a problem, as novel *Flavobactericeae* species are described.

Three species-specific primers were used initially; PSY1 – PSY2 for *F. psychrophilum* (105), Sol-Fw – Sol-Rv for *T. soleae* (109) and Mar1 – Mar2 for *T. maritimum* (106). When DNA of reference strains and Icelandic sample isolates was amplified with these published species-specific primers, some species-non-specific amplification was observed (Figure 4).

Comparison of 16S rRNA gene sequences from Icelandic fin and tail rot bacterial isolates and published sequences from other countries resulted in the design of new species-specific primers for the detection of *F. psychrophilum, T. maritimum, T. soleae, F. columnare* and *F. branchiophilum,* targeting the 16S rRNA gene and the 16S – 23S IGS region.

Despite the predicted specificity of the new primer pairs, some species-non-specific amplification was observed (Tables 5 and 6). The specificity of the primers was tested using five bacterial strains; commercially available *F. psychrophilum, T. maritimum* and *T. soleae* reference strains as well as *F. branchiophilum* and *F. columnare* isolates, generously provided by the Department of MCB at the University of Guelph. For optimum PCR specificity, touchdown PCR was used and the PCR plate was kept on ice until the thermal cycler had reached 80°C. The results indicate that two primer pairs were species-specific: primer pair 8 (*F. psychrophilum* (Figures 8 and 9)) and primer pair 17 (*T. maritimum* (Figure 5)) (Table 7). However, the primers need to be tested against more bacterial isolates and amplification conditions need further to be optimized to confirm the results.

Species-specific primers for tail and fin rot bacteria have also been designed to target the *gyrB* and 16S – 23S IGS regions. In this study, primer pairs targeting the *gyrB* of *F. psychrophilum* (primers: PSY-G1F – PSY-G1R) and 16S – 23S IGS region of *T. soleae* (primers: G47F – G47R) were tested.

When the *gyrB* from reference strains was amplified, first using universal *gyrB* primers and then various dilutions of the PCR product for species-specific PCR using the PSY-G1F – PSY-G1R primer pair (*F. psychrophilum* specific), some species-non-specific amplifications were observed. The results indicate that this primer pair is unsuitable for use. When the 16S – 23 IGS region was amplified from reference strains using the G47F – G47R primer pair (*T. soleae* specific) with gradually decreasing annealing temperature, some species-non-specific amplifications were observed. The results were promising, however, because with increased annealing temperature, the species-non-specific amplification products decreased. In order to improve further the specificity of the G47F – G47R primers, we varied the $MgCl_2$ concentration of the reaction, used touchdown PCR and two concentrations of DNA. This resulted in *T. solea* specific amplification (Figure 9).

The use of species-specific primers may not be a feasible solution for differentiating *Flavobacterium* and *Tenacibaculum* species. With increasing number of primer pairs targeting the 16S rRNA gene, the more likely it is that primers cross react with DNA from other species. For pathogen detection, primers targeting either plasmid DNA or virulence factors may be a possible solution. Furthermore virulence genes can be used to discriminate between pathogenic bacteria and harmless ones (81).

DNA sequencing of PCR product using 16S rRNA gene universal PCR primers may also be an alternative solution for reliable identification of tail and fin rot pathogens as used in this study. DNA sequencing can also be used to reveal new mutations and variants (81).

5.2 Genetic relationship

Evolutionary trees display key information such as diversity, origin and pattern of ancestry by merging branches at points representing common ancestors, called nodes. Each branch represents an isolate sequence or sequence for a group of isolates that connect through internal nodes of more distant ancestors shared by two or more lineages (137). When a group of biological taxa or species share features inherited from a common ancestor, the group is referred to as a clade (138).

To show distance-based relationships, the neighbor-joining method was chosen because it can rapidly analyze large datasets. Evolutionary trees based on other algorithms were created as well for comparison (Appendix III).

MUSCLE alignment was used to construct sequence alignments in this study. MUSCLE alignment uses a matrix-based algorithm where sequences are aligned using pairwise alignments to create a distance matrix, which is then used to create a phylogenetic tree. Distance matrixes are clustered using UPGMA, which is a simple “bottom up” hierarchical clustering method in the MUSCLE alignment where each sequence starts as an individual cluster, the clusters are then paired and merged as one moves up the hierarchy. UPGMA method gives good accuracy between two sequences by aligning the ones that have fewest differences together, even if they are not evolutionary neighbors (139).

The neighbor-joining method can be used to calculate phylogenetic trees from evolutionary distance data using pairs of operational taxonomic units (OTUs). Pairs of OTUs are related clades and can be referred to as neighbors and are connected through a single node. OTUs are used to minimize

the total branch length of a cluster, starting with a star-like tree from that point where there is no clustering of OTUs. The sum of branch length is calculated for all pairs of OTUs and the pair with the lowest branch length is selected as a pair of neighbors which then is used to form an unrooted, bifurcating tree which has exactly two descendants arising from a node (140).

Bootstrap methodology is used to test whether the shortest tree (a tree that requires the fewest evolutionary changes) is a reliable model for phylogenic relationship or if it varies from other short trees by chance. Bootstrapping is a procedure where variables, such as nucleotides, are randomly "sampled" to give a certain phylogenetic analysis. Then the phylogenetic data sampling is repeated and eventually gives an estimate of confidence for each clade of a phylogenetic tree, based on the percentage of bootstrap trees showing the same clade. The higher the bootstrap percentage value, the higher is the confidence level for the position of each clade within a phylogenetic tree (138, 141).

In the bootstrap consensus tree (Figure 10B), relatively low bootstrap values are displayed for many of the larger nodes. This can be caused by the large amount of sequences used to generate the tree, where groups of branches connected by a node may be frequently found at the same place in the tree (142). Larger bootstrap values for other nodes indicate that the branches within that node show a close relationship with good confidence.

Other types of phylogenetic trees were calculated using maximum likelihood, maximum parsimony, UPGMA method and minimum evolution method (Appendix III). These methods give very similar results as the neighbor-joining method, only differing in position of clades. Only a few variations are noticeable within each clade for various trees.

It can be difficult to interpret similarity or dissimilarity, to define species and genera, using 16S rRNA gene sequences. The results can vary if they are calculated using different sizes of fragments and the choice of algorithm can also influence the results. Similarity in the 16S rRNA gene sequences between genera can also complicate the analysis. Generally, 5% dissimilarity or more is used as a standard for a new genus definition. Values of 5% or less dissimilarity can either be considered a species, sub-species or genera, depending on the standard of limits (101).

The phylogenetic trees using MUSCLE alignment and neighbor-joining analysis revealed the genetic relationship of Icelandic tail and fin rot isolates and commercially available *F. psychrophilum, T. maritimum* and *T. soleae* reference strains (Figure 10A). Using this analysis *F. psychrophilum* reference strain and approximately 30% of the Icelandic isolates (groups H21 and H22) show 98% similarity. Furthermore, the phylogenetic relationship shows that even though the Icelandic Flavobacterium strains show high similarity, their 16S rRNA genes are different. Therefore the choice of a single isolate for vaccine development would be of concern.

In this study most water samples cluster together at the top of the tree that may exclude infection via vertical transmission. The roe samples on the other hand seem to cluster more closely to samples taken from diseased fish (Figure 11). The possible route of transmission in the tail and fin rot diseases has been debated and a conclusion has not yet been reached. Further investigation using more roe samples and bigger volumes of intake water from fish farms may lead us closer to the answer.

5.3 Bacterial growth

In this part of the study, the goal was to find optimal growth conditions for tail and fin rot reference strains and bacterial isolates from Icelandic isolates that appear to be closely related to the reference strains. A heat tolerance study was carried out to find at what temperature optimal bacterial growth rate was obtained. Such information is essential for future studies like challenge studies. A salinity test was performed to examine whether bacteria could grow at different salt concentrations. We wanted to examine the chances of bacteria, originally isolated from one salt and temperature condition, being able to infect fish in another environment and salinity. However, it has to be kept in mind that our experiments were performed *in vitro* and the results are not directly transferrable to an *in vivo* system.

The temperature and salinity tolerance tests were performed on reference strains for *F. psychrophilum*, *T. maritimum* and *T. soleae* as well as Icelandic bacterial isolates. *F. psychrophilum* is a slow growing freshwater bacterium that thrives at relatively cold temperatures (13, 28) while *T. maritimum* and *T. soleae* have been isolated from diseased fish at relatively high sea temperature (46, 56).

In the salinity tolerance test, each reference strain and isolate was cultured in FMM medium with one of three different salinity levels and the bacterial density measured for five consecutive days (Figures 13 and 15). A two-factor ANOVA test, which determines differences between the means of independent groups, showed a significant effect of salinity on bacterial density, $p<0.0001$ for all reference strains. As predicted, FMM medium containing freshwater (0 ppm salt) was best suited for *F. psychrophilum* growth while *T. maritimum* and *T. soleae* grew best in FMM medium containing seawater (32 ppm salt), with *T. soleae* showing significantly more rapid growth, $p<0.0001$. No growth was detected for the two bacterial strains at 0 ppm salinity (freshwater). However, both *T. maritimum* and *T. solea* were able to grow at 16 ppm (brackish water), which indicates more salinity tolerance than was predicted.

Freshwater medium (0 ppm) was best suitable for Artic charr isolates #2 and #6 and Atlantic salmon isolates #1 and #2. Medium containing brackish water (16 ppm) was more suitable for Artic charr isolates #3 and #7 and culture in medium containing seawater (32 ppm) was most suitable for lumpfish, rainbow trout, haddock and redfish isolates.

Out of freshwater bacteria isolates (Artic charr and Atlantic salmon isolates) Artic charr isolate #3 and #6 were able to grow on medium containing seawater (32 ppm) likely because the isolates have more salinity tolerance than other Artic charr isolates tested.

Artic charr isolates #2 and #3 fall within group H21 according to the neighbor-joining analysis, but this group is closely related to the *F. psychrophilum* reference strain (Figure 10A). Isolate #3 was able to grow on medium containing seawater like *F. psychrophilum,* however, isolate #2 did not grow on seawater medium. Artic charr isolate #6 did grow on seawater medium and falls within group H22. Only testing more isolates within this group, will help us understand if high salinity tolerance is a characteristic for this group.

According to our analysis Artic charr isolates #2 and #3 have identical 16S rRNA gene, it is however very likely that genes controlling salinity tolerance vary between these isolates. It has been

stated that *F. psychrophilum* is unable to grow under seawater circumstances (143), but our study shows that *F. psychrophilum* is able to grow on medium containing seawater (Figure 10A). This finding can possibly help solve the question regarding whether *F. psychophilum* can survive migration to sea and cause infection when fish returns to freshwater to spawn (143).

The ability of isolates to grow on medium containing brackish water raises questions about whether freshwater fish carrying BCWD infection is able to infect seawater fish living on river mouths.

The optimal temperature for *F. psychrophilum* and the Icelandic isolates was 15°C which matches published optimal temperature for *F. psychrophilum* but it ranges between 15°C and 20°C (13, 28). *T. maritimum* and *T. soleae* grow best at 15°C with their optimal temperatures ranging between 22°C and 30°C (3, 45-47, 56). Although high temperatures are best suited for tail and fin rot reference strains, detectable growth was at 5°C and 10°C for *F. psychrophilum*, *T. soleae* and the Icelandic isolates indicating that growth can be readjusted to environmental conditions in Iceland. *Moritella viscosa* has also been tested in regards to temperature, but *M. viscosa* seems to become more unstable above 10°C and that could be a reason for that the bacteria is unable to infect fish at higher temperatures (144).

Treatment at hypo-salinity level, generally around 12 ppm to 16 ppm, is a popular method for disinfecting marine fish with the *Cryptocaryon irritans* parasite. The parasite causes conditions referred to as “saltwater itch” (145). However, there is no evident use of hypo-salinity treatment for treating fish with bacterial diseases, although some attempts have been made. In fish farms, great care is required in salinity adjustments due to higher environmental stress that can be an important factor in outbreaks of infectious diseases of fish (146). This experiment demonstrates that hyper salinity is likely to be an insufficient treatment for flavobacterial diseases.

When Icelandic *Flavobacterium* isolates were chosen for the salinity and temperature tolerance study a few isolates did not grow on liquid FMM medium. An interesting aspect is whether this can possibly affect bacterium virulence and host specificity. Bacterial adhesion has been associated with the virulence of *F. psychrophilum.* Furthermore, adhesion to gill tissue has been proposed to be a passage for the bacterium into host tissue and may give direct toxin affection (147). Although *F. psychrophilum* lack pili and flagella, they feature cell surface proteins that are considered to be adhesion molecules. The bacterium then uses these adhesion molecules to recognize host cell surface and bind its victim. Expression of adhesion molecules may affect the ability of the bacteria to grow in liquid FMM medium. A subset of Icelandic *F. psychrophilum* bacteria were not able to grow in liquid medium, and it would be interesting to examine whether this translates into an effect on virulence of the isolates (148, 149).

6 Conclusion

The 16S rRNA genetic relationship of Icelandic tail and fin rot isolates and *F. psychrophilum*, *T. maritimum* and *T. soleae* reference strains reveals that *F. psychrophilum* isolates can be found in Iceland. Furthermore, the phylogenetic tree indicates that transmission from water to fish is unlikely, as bacteria sampled from water and diseased fish did not overlap.

PCR primer design requires target DNA that is variable between species but has highly conserved regions within species. Furthermore, highly related species should be included as negative controls when testing the specificity of designed primers to exclude species-non-specific amplification.

Identification of Icelandic *Flavobacterium* and *Tenacibaculum* isolates using published species-specific PCR primers resulted in species-non-specific amplification. New designed species-specific primers revealed that the 16S rRNA gene and the 16S – 23S IGS region might not be suitable for *Flavobacterium* and *Tenacibaculum* species identification.

Rapid identification of tail and fin rot bacteria is important for routine diagnosis and vaccine development. For a rapid detection of tail and fin rot bacteria by PCR, the choice of PCR primers and amplification conditions need to be optimized. Another solution for a reliable diagnosis is using DNA sequencing, which is still, however, more time consuming and more expensive than using PCR amplification.

Bacterial growth, measured in a salinity tolerance experiment, indicates that hypo-salinity is not an ideal treatment for fish with tail and fin rot as bacterial growth was measurable for *F. psychrophilum, T. maritimum* and *T. soleae* reference strains in FMM using 16 ppm, resembling brackish water. All Icelandic *Flavobacterium* isolates that were cultured in liquid FMM medium can grow in brackish environment (16 ppm) suggesting that transmission of infection is possible from fish cultured in freshwater to wild/reared fish at sea by special environments like river mouths. Results from the temperature tolerance experiment suggest that environmental conditions in Icelandic oceans can favor flavobacterial disease outbreaks and that tail and fin rot occurrences need to be monitored, especially in seawater farmed salmon aquaculture.

Future studies might involve advanced genetic research on tail and fin rot isolates using multilocus sequence typing (MLST) technique for better understanding of isolate placement within the phylogenetic tree. Furthermore, genome sequencing on *F. psychrophilum* virulence factors would be a foundation for vaccine development.

BCWD has already become established in Icelandic aquaculture, and is likely to have an increased impact on fish health as aquaculture continues to grow in Iceland. To control or prevent disease, fish farmers and scientists need to be aware of the infection risks and continue to work together to minimize the impact of the bacterium.

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Appendix I

Buffers and solutions

Flexibacter maritimus medium (FMM)

5% Peptone, 0,5% Yeast extract, 0,01% Sodium acetate, pH 7,2 - 7,4

FMM was made with double distilled water ddH_2O for culturing freshwater samples. Culturing of saltwater samples was with FMM made of aged seawater filtered through 185mm Whatman™ filter paper (GE Healthcare).

Phosphate-buffered saline PBS

5% NaCl, 1,79% Na_2HPO_4 x $12H_2O$, 0,125% KCl, 0,525% KH_2PO_4

Minimal salt solution (MSS)

24% NaCl, 0,7% $MgSO_4$, 0,075% KCl

5x Tris borate-EDTA (TBE) buffer

0,045 M Tris borate, 0,0001 M EDTA

10x Restriction buffer (RSB)

50% glycerol, 15 mM EDTA, 0,25% bromophenol blue

Appendix II

Primers used in this study

16S rRNA universal

8F	5'-AGA GTT TGA TCC TGG CTC AG-3'
1544R	5'-AGA AAG GAG GTG ATC CAG CC-3'
805R	5'-GGA TTA GAT ACC CTG GTA GTC-3'

F. psychrophilum

16S rRNA gene

PSY1	5'-GTT GGC ATC AAC ACA CT-3'
PSY2	5'-CGA TCC TAC TTG CGT AG-3'
PSYF (unpublished)	5'-CGT AGT GGC TGC TCT CTG TAC C-3'
PSYR (unpublished)	5'-CCA GAT AAG TCA GTG GTG AAA GC-3'

Gyrase B gene

GYR1	5'-CAY GCN GGN GGN AAR TTY GA-3'
GYR1R	5'-CCR TCNACR TCN GCR TCN GT-3'
PSY-G1F	5'-TCG AGG AAA TCT TAC ACT CG-3'
PSY-G1R	5'-GTT GCA ATT ACA ATG TTG T-3'

Intergenic spacer region (unpublished)

Psy-3F	5'-TAG GGG TCG ACA GTT CGA GT-3'
Psy-3R	5'-CAG CTT ATC ACG CCC TTC AT-3'
Psy-4F	5'-ATG TAG GGG TCG ACA GTT CG-3'
Psy-4R	5'-GCT TTT CGC AGC TTA TCA CG-3'
Psy-5F	5'-TTG AGG ATT CAA CCA AAA GC-3'
Psy-5R	5'-ATC CCC CAT ACG CCC TTA TT-3'

T. maritimum

16S rRNA gene

Mar1	5'-TGT AGC TTG CTA CAG ATG A-3'
Mar2	5'-AAA TAC CTA CTC GTA GGT ACG-3'
MarF (unpublished)	5'-CCT ACG AGT AGG TAT TTG ACG GTA-3'
MarR (unpublished)	5'-GTT TGC ACC GGC AGT CTC-3'

Intergenic spacer region (unpublished)

Mar-1F	5’-CAG TCT CGT AGC TCA GCT GGT-3’
Mar-1R	5’-GTT CAG ATT ATA AAA TCC TCA ATG C-3’
Mar-2R	5’-TGA GCT AAT CCC CCA TAT GAA-3’

T. soleae

16S rRNA gene

SOLFW	5’-TGC TAA TAT GTG GCA TCA CAA-3’
Sol-Rv	5’-CAA CCC ATA GGG CAG TCA TC-3’
SolF (unpublished)	5’-GGT CGC TCC TCT CGG TAA-3’
SolR (unpublished)	5’-CGA TGG ATA CTA GTT GTT GGG TTA-3’

Intergenic spacer region

G47F	5’-ATGCTA ATA TGT GGC ATC AC-3’
G47R	5’-CGT AAT TCG TAA TTA ACT TTG T-3’

F. columnare

16S rRNA gene

ColF	5’-CAG TGG TGA AAT CTG GT-3’
ColR	5’-GCT CCT ACT TGC GTA GT-3’
ColF (unpublished)	5’-TTT TCA GAT GGC CTC ATT TG-3’
ColR (unpublished)	5’-AAA CGT CCG AAG AAA GAT CTG-3’

Intergenic spacer region (unpublished)

Col-1F	5’-GGTCCG TAG GCG GTT TTA T-3’
Col-1R	5’-AGG TAC CCC CAG CTT CCA T-3’
Col-2R	5’-CCG TAG GCG GTT TTA TAA GTC A-3’

F. branchiophilum

16S rRNA gene

BraF (unpublished)	5’-TGT GAT GAT CGC ATG GTT TTC AC-3’
BraR (unpublished)	5’-CGT CAA GCT TCT ACT CGT AGA AGT G-3’

Appendix III

Phylogenetic trees

A **B**

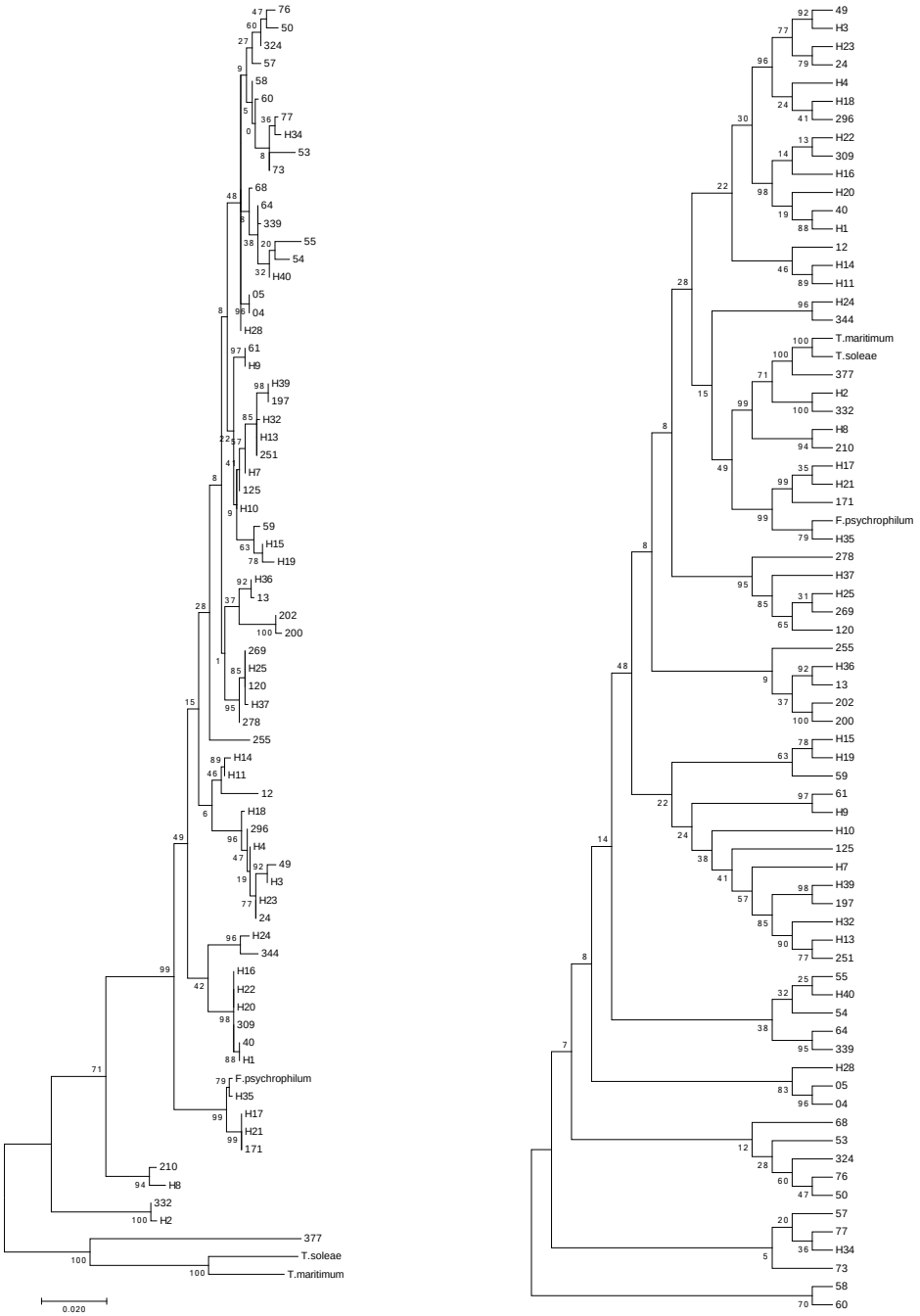

Figure 16. Relationship within Icelandic *Flavobacterium* strains using the maximum likelihood method. Shown is the relationship based on 16S rRNA sequence analysis using the maximum likelihood method with 500 bootstrap replications. The tree displayed is with the highest log likelihood (-5373.5569). Single strains are represented with a number and a group of strains is represented with a H-name. The scale bar is 0.02. The numbers by the branches show the confidence limits estimated by bootstrap analysis. A: Optimal tree with bootstrap values B: Bootstrap consensus tree with no cutoff.

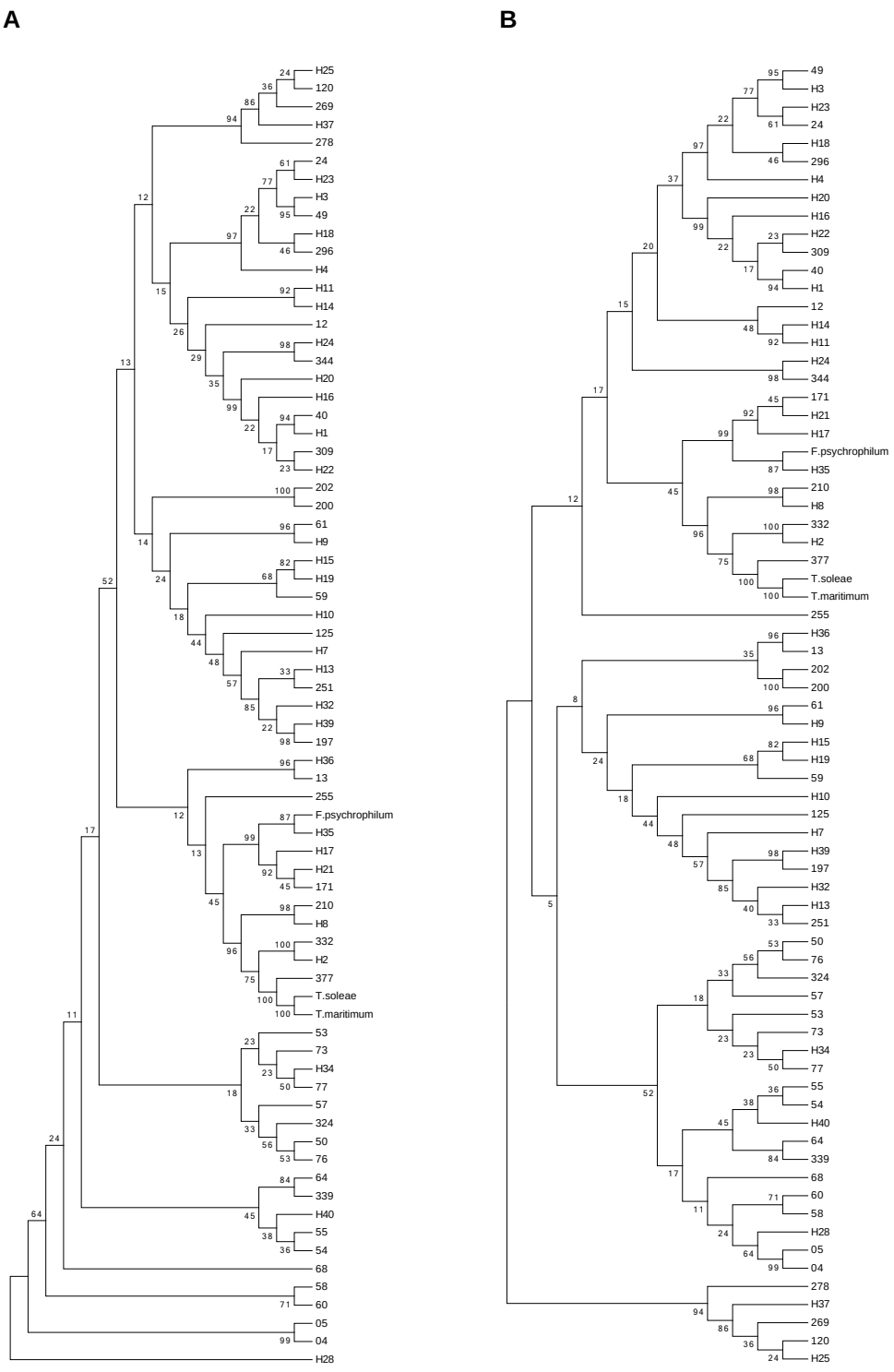

Figure 17. Relationship within Icelandic *Flavobacterium* strains using maximum parsimony analysis. Relationship based on 16S rRNA sequence analysis using maximum parsimony analysis with 500 bootstrap replications. The most parsimonious tree with length: 598 is shown. The consistency index is 0.463, the retention index is 0.782 and the composite index is 0.403 for all sites. Single strains are represented with a number and a group of strains is represented with a H-name. The numbers by the branches show the confidence limits estimated by bootstrap analysis. A: Optimal tree with bootstrap values B: Bootstrap consensus tree with no cutoff.

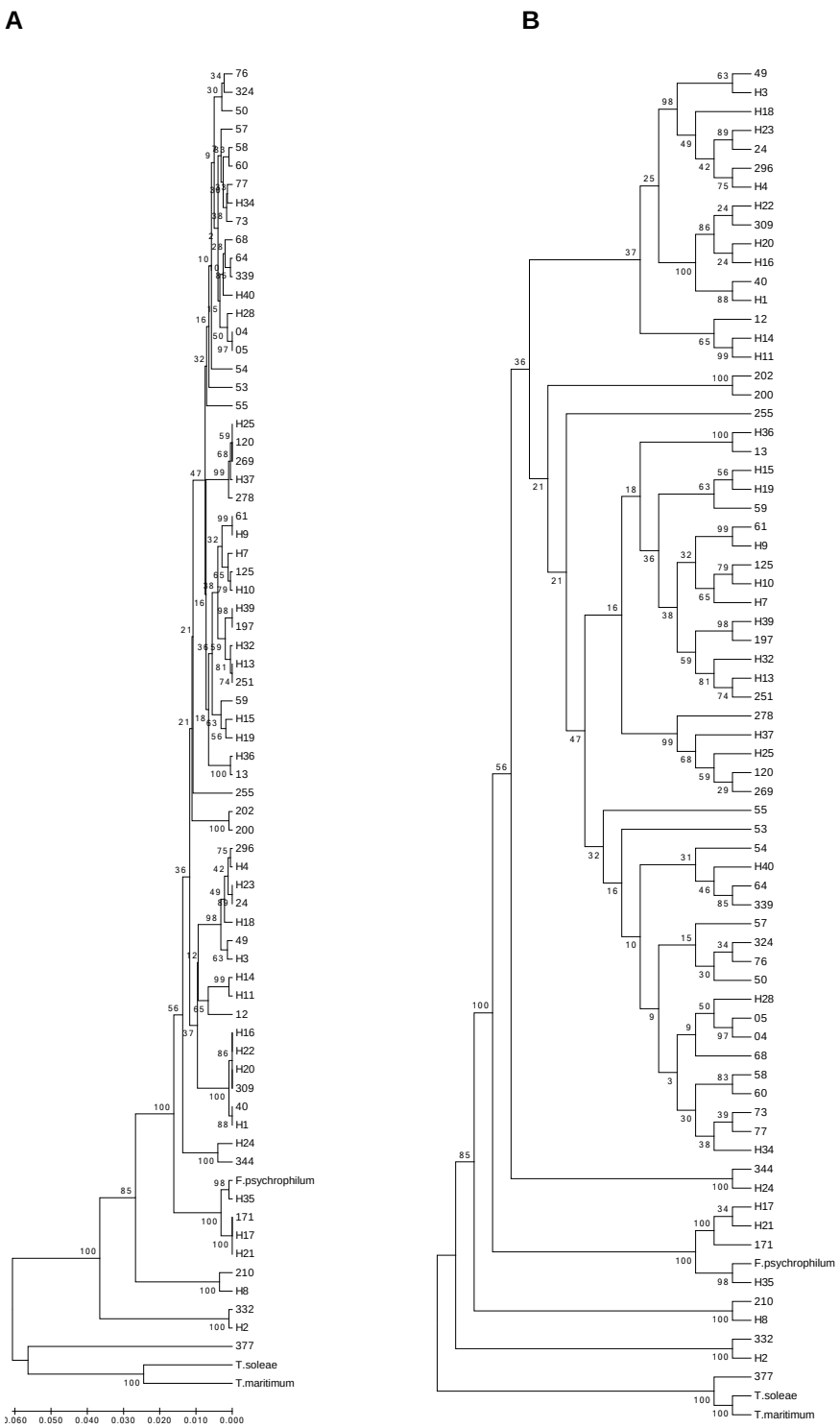

Figure 18. Relationship within Icelandic *Flavobacterium* strains using UPGMA method. Relationship based on 16S rRNA sequence analysis using UPGMA method with 500 bootstrap replications. Single strains are represented with a number and a group of strains is represented with a H-name. The optimal tree with the sum of branch length: 0.473 is shown. The numbers by the branches show the confidence limits estimated by bootstrap analysis. A: Optimal tree with bootstrap values B: Bootstrap consensus tree with no cutoff.

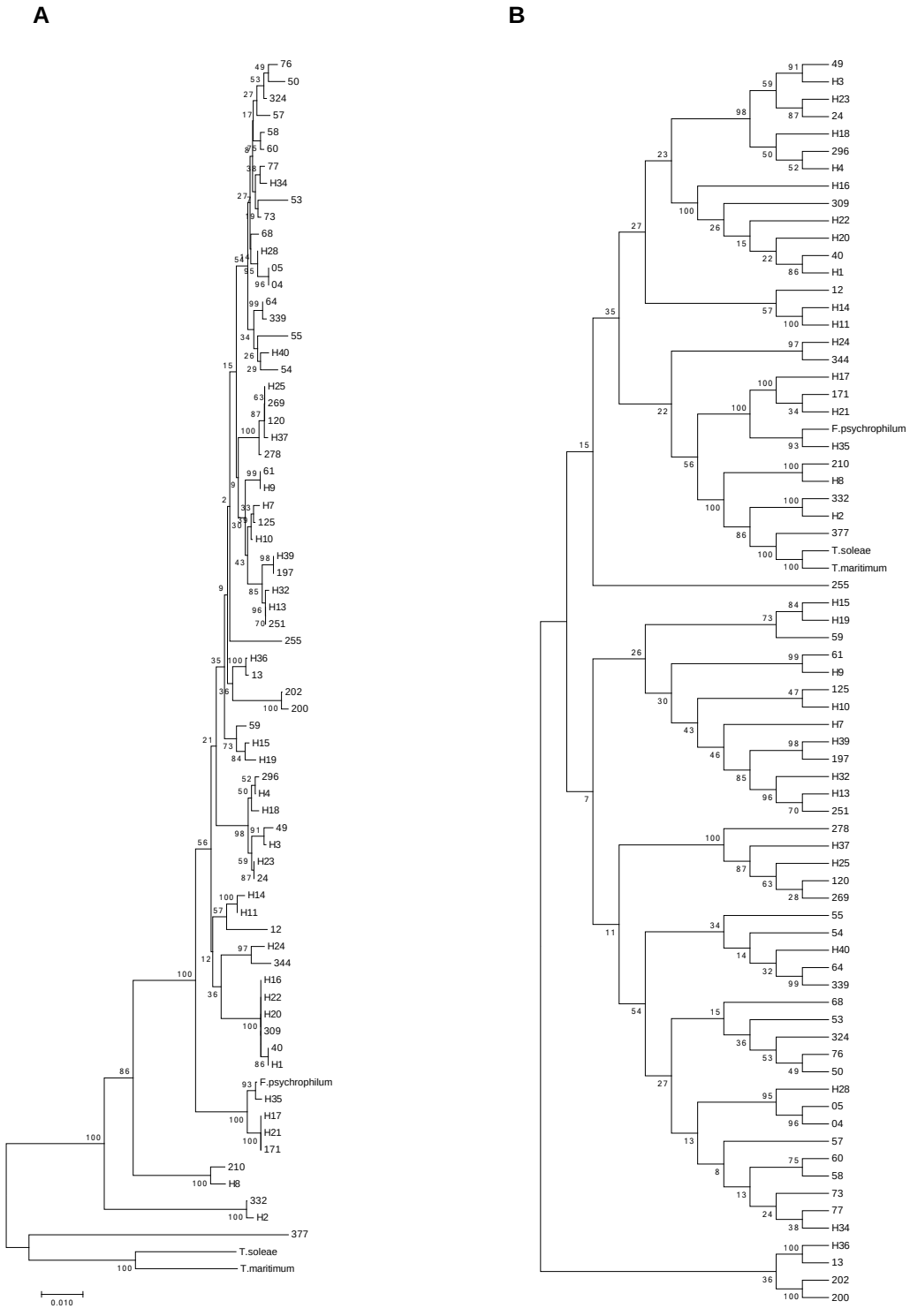

Figure 19. Relationship within Icelandic *Flavobacterium* strains using minimum evolution method. Relationship based on 16S rRNA sequence analysis using minimum evolution method with 500 bootstrap replications. The optimal tree with the sum of branch length: 0.472 is shown. Single strains are represented with a number and a group of strains is represented with a H-name. The scale bar is 0.01. The numbers by the branches show the confidence limits estimated by bootstrap analysis. A: Optimal tree with bootstrap values B: Bootstrap consensus tree with no cutoff.

Table 8. Phylogenetic tree construction settings. NR = non relevant

	Maximum-likelihood method	Neighbor-joining	Minimum Evolution	UPGMA	Maximum Parsimony
Analysis	Phylogeny reconstruction	Phylogeny reconstruction	Phylogeny reconstruction	Phylogeny reconstruction	Phylogeny reconstruction
Statistical method	Maximum Likelihood	Neighbor-joining	Minimum Evolution method	UPGMA	Maximum Parsimony
Phylogeny test					
Test of phylogeny	Bootstrap method	Bootstrap method	Bootstrap method	Bootstrap method	Bootstrap method
No. of bootstrap replications	500	500	500	500	500
Substitution model					
Substitution type	Nucleotide	Nucleotide	Nucleotide	Nucleotide	Nucleotide
Model/method	Tamura-nei model	Maximum composite likelihood	Maximum composite likelihood	Maximum composite likelihood	NR
Substitutions to include	NR	d: Transitions and transversions	d: Transitions and transversions	d: Transitions and transversions	NR
Rates and patterns					
Rates among sites	Uniform rates	Uniform rates	Uniform rates	Uniform rates	NR
No. of discrete gamma categories	Not applicable	Not applicable	Not applicable	Not applicable	NR
Pattern among linages	NR	NR	Same (homogeneous)	Same (homogeneous)	NR
Data subset to use					
Gaps/missing data treatment	Complete deletion	Complete deletion	Complete deletion	Complete deletion	Complete deletion
Site coverage cutoff (%)	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Tree inference options					
ML heuristic method	Nearest-neighbor-interchange (NNI)	NR			NR
Initial tree for ML	Make initial tree automatically (Default-NJ/BioNJ)	NR			NR
ME heuristic method	NR	NR	Close-neighbor-interchange (CNI)		NR
Initial tree for ME	NR	NR	Obtain initial tree by neighbor-joining		NR
Initial tree file	Not applicable	NR			NR
ME search level	NR	NR	1		NR
MP search method	NR	NR	NR	NR	Subtree-pruning-regrafting (SPR)
No. of initial trees (random addition)					10
MP search level					1
Max no. of trees to retain					100
Branch swap filter	None	NR			
System resource usage					
Number of threads	1	NR			

Table 9. Detailed distribution of isolates used in phylogenetic relationship tree.

Artic charr roe	Water		Artic charr			Atlantic salmon	Rainbow trout	Lumpfish	Redfish
24	1	91	3	79	148	39	344	377	126
25	11	92	4	80	149	40			127
26	49	93	5	81	150	41			336
27	50	120	6	82	151	42			337
251	51	121	7	83	157	43			338
252	52	122	8	84	158	44			
253	53	123	9	99	159	45			
254	54	125	10	100	160	46			
255	55	152	12	101	161	47			
256	56	153	13	102	164	48			
257	57	154	14	103	168	68			
258	58	193	15	104	171	69			
259	59	194	16	105	332	70			
308	60	195	18	106	332	71			
309	61	196	19	107	333	72			
311	62	197	20	109	334				
312	64	198	21	110	335				
314	65	199	22	111					
315	66	200	23	113					
316	67	201	28	114					
317	73	202	29	115					
318	74	204	30	116					
319	75	205	31	117					
320	77	207	32	118					
324	85	210	33	119					
329	87	277	33	133					
330	88	278	34	138					
331	89	321	35	143					
	90	339	36	145					
			37	146					
			38	147					